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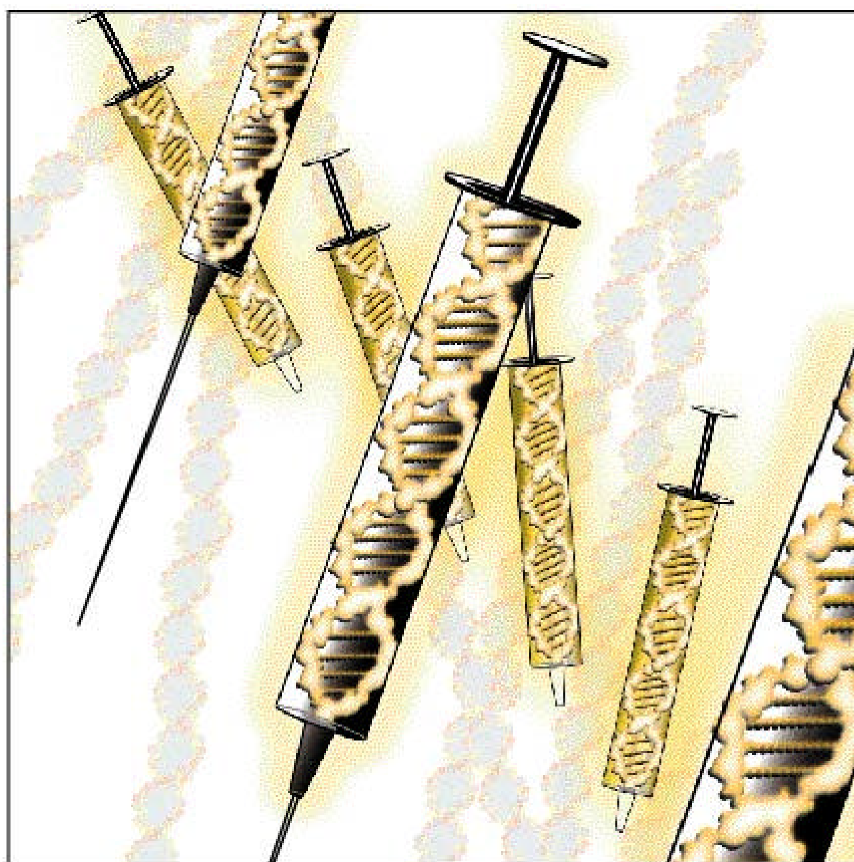
An Orphan in Science: Environmental Risks of Genetically Engineered Vaccines



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TRONDHEIM, NORWAY

Directorate for Nature Management
7485 Trondheim, Norway
Telephone: +47 73 58 05 00 - Telefax: +47 73 58 05 01
http://www.naturforvaltning.no

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An Orphan in Science: Environmental Risks of Genetically Engineered Vaccines.

Author:

Terje Traavik, Departments of Virology and Medical Microbiology, University of Tromsø; GENØK-Norwegian Institute of Gene Ecology, Tromsø, Norway

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Ekstrakt:

Moderne molekylær biologi, rekombinant DNA teknologi og genteknologi har åpnet opp for en rekke alternative strategier for vaksine produksjon. Utredningen tar for seg de ulike strategiene og ser på mulige risikofaktorer og farer knyttet opp mot disse**.**

Abstract:

Modern molecular biology, recombinant DNA technology and genetic engineering have opened the road to a number of alternative strategies for vaccine production. This research report deals with the different strategies and tries to analyse risk factors and potential hazards.

Foreword

The background for this project is the Directorate's management tasks in connection with modern biotechnology and responsibility for the nature environment. The Gene Technology Act regulates, among other things, the release of genetically modified organisms (GMOs) into the environment. When an application is made to release a GMO, an environmental impact assessment report has to be prepared dealing with possible health hazards and risks to the environment. In that context, there are many unanswered questions.

Modern molecular biology, recombinant DNA technology and genetic engineering have opened the road to a number of alternative strategies for vaccine production. Most research take place in the industry and the biomedical sphere and almost none in relation to environmental research.

This report was initiated on account of the lack of knowledge regarding environmental question relating to genetically engineered vaccines. The aim was to throw more light on the problem and offer recommendations to the environmental management authorities about the future research.

The entire project has been in the hands of Professor Terje Traavik at the University of Tromsø, GENØK-Norwegian Institute of Gene Ecology, on behalf of the Directorate for nature Management.

Trondheim, September 1999

Yngve Svarte
Director of the Department for Species Management

Preface

This report has been written on an assignment from the Norwegian Directorate of Nature Management. Its title provides its final and most clear-cut conclusion: from an ecological and environmental point of view many first generation live, genetically engineered vaccines are inherently unpredictable, possibly dangerous, and should not be taken into wide-spread use until a number of putative problems have been clarified. Only targeted scientific approaches can contribute to clarification and, hopefully, elimination of such problems.

It has been hard work to arrive at the finishing line of this project. There is no risk-associated scientific literature to lean on, or interpret from. Consequently, I have had to rely to a very high extent on my own insight and imagination, and root them in the "Precautionary principle". I can clearly see that some readers might call my extrapolations and analyzes far-fetched and without scientific basis. This may well be the case, but, in addition to my own inadequacy, that only reflects the reality of writing about "a scientific orphan".

I sincerely thank the Directorate of Nature Management for assigning this project to me. The ambitions and workload grew larger than the allotted time permitted. I am very grateful to the Directorate, represented by Anne Britt Storeng and Hilde Christin Larsen, for awaiting the overdue product in a patient and graceful manner.

Good colleagues and friends have given me valuable advice, criticism and encouragement. I particularly want to mention my coworkers at GENÔK-Norwegian Institute of Gene Ecology: Drs. Dag Coucheron, Steinar Johansen and Örjan Olsvik; and Dr. Mae-Wan Ho of the Open University in UK.

Tromsø, Norway/Penang, Malaysia in June 1999.

Terje Traavik

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Summary

This report is approaching potential ecologic and environmental risks posed by some types of genetically engineered or modified vaccines that are now being developed, and may soon be in widespread use. The risks and hazards discussed are most certainly within the realm of possibility, and according to the precautionary principle they should be subject to preventive measures. In practice, however, the risks are non-existent from medical and scientific points of view, since they have not been supported by experimental or epidemiological investigations. This, again, is a "Catch-22" situation, in the sense that such investigations have not been performed at all.

The main purpose of the report is to raise awareness and catalyze discussions. If this in its turn may contribute to having resources available for public funding of independent research, the efforts of the author have been well rewarded.

Chapter 1 sets the stage by briefly reviewing some fundamental conceptions. Vaccination is a form of prevention or prophylaxis of infectious disease and cancers. The reasons for giving priority to prevention and prophylaxis are stronger than ever, as development of resistance in microorganisms, viruses and cancer cells are reducing the therapeutic opportunities offered by chemotherapeutics and antibiotics.

Through their continual battle with microorganisms and viruses, vertebrates have evolved an elaborate set of protective measures collectively termed the *immune system*. Infection with a specific disease agent may initiate immunity to that agent, and an individual that is immune to a specific infectious agent will be left unharmed when infected by that agent again.

Vaccination intends to provide individuals with immunological protection before an infection actually takes place. But the immune system is very complex, and immunity against different infectious agents is based on fine-tuned balances between the various types of cells, signal substances and antibodies that make up the total immune system. For some disease agents cellular immune reactions are more important, for others specific antibodies are essential for protection. Because vaccination against a threatening disease may take place many years before exposure to the disease-causing agent, immunological memory is a critical factor. A long-lived immune response that may be mobilized and augmented rapidly when called for, is essential. Furthermore, local immunity on the epithelial surfaces that are the portals of entrance to the body for most infectious agents, is very important.

Until recently most *traditional vaccines* were of the "whole disease agent"-type: after varying degrees of purification the whole bacterial cell or virus particle was used for immunization. Such vaccines might be killed, inactivated or "live".

By modern techniques "killed" vaccines may be based on single proteins purified extensively to constitute safe preparations with seemingly no side effects. But in general such vaccines have given short-lived general immune responses, and weak local immune responses. This may, however, be due to rather crude and inadequate delivery systems for such vaccines.

Live vaccine agents infect the vaccinees, but have had their disease provoking abilities attenuated. “Live” vaccines often give stronger mobilization of all effector parts of the immune system, and in many instances also good local immunity. The most prominent drawback of such vaccines is that they may revert to their full disease-causing potential.

The very short *Chapters 3 and 4* define important concepts in the context of risk assessment.

Chapter 2 summarizes the strategies used to achieve various types of vaccines by recombinant DNA techniques and genetic engineering, while *Chapter 5* is devoted to a discussion of risks and hazards related to the different alternatives.

Synthetic and recombinant vaccines are produced under contained conditions, and only a polypeptide which may confer protective immunity to a given disease agent are brought out of the production unit and used as vaccine. Such vaccines carry the same advantages and disadvantages as traditional “killed” or “subunit” vaccines. It is conceivable that new vaccine delivery systems and basic knowledge about immune system interactions will make these vaccines more efficient in the near future. It is difficult to imagine such vaccines posing ecologic and environmental risks.

Genetically modified viruses and genetically engineered virus-vector vaccines carry significant unpredictability and a number of inherent harmful potentials and hazards. The immunological advantages of such vaccines are related to the fact that the viruses are “live” and infect the vaccinated individuals. It has, however, been demonstrated that minor genetic changes in, or differences between, viruses can result in dramatic changes in host spectrum and disease-causing potentials. For all these vaccines important questions concerning effects on other species than the targeted one are left unanswered so far. The opportunity of a genetically engineered vaccine virus to engage in genetic recombinations with naturally occurring relatives is another unpredictable option. The new, hybrid virus progenies resulting from such events may have totally unpredictable characteristics with regard to host preferences and disease-causing potentials. Furthermore, when genetically modified or engineered virus particles are broken down in the environment, their nucleic acids will be released, representing the same unpredictable risk potentials as the DNA and RNA vaccines discussed below.

Much basic work is needed before recombinant bacterial vectors may be taken into practical use. For instance, it was recently demonstrated that genetically engineered bacteria might transfer their new gene efficiently to indigenous bacteria in the mamalian gut. This potential risk has not been investigated for bacteria that are now being genetically engineered as oral vaccines.

Naked DNA vaccines are engineered from general genetic shutle vectors. They are constructed to break species barriers. Naked DNA may persist much longer in the environment than dogmas held just a short time ago. Consequently, upon release or escape to the wrong place at the wrong time, horizontal gene transfer with unpredictable long- and short-term biological and ecological effects is a real hazard with such vaccines. There is also growing concern about harmful effects due to random insertions of vaccine constructs into cellular genomes in target or non-target species.

RNA vaccines may have a far way to go before any of them find practical use. Although easy degradation is a serious problem with RNA work in the lab, RNA may be surprisingly resistent

under natural conditions. At the present time recombination between related RNA molecules has become a real concern. RNA recombination is far more common than dogmatic views held until recently.

"Edible vaccines" are produced by genetically engineered plants. Little is known about the consequences of releasing such plants into the environment, but there are examples of transgenic plants that seriously alter their biological environment. A number of unpredicted and unwanted incidents have already taken place with genetically engineered plants.

Chapter 6 first discusses some special considerations with regard to how some environmental pollutants (xenobiotics) may interact with genetically engineered vaccines. It then goes on to specific problems related to the use of such vaccines within aquaculture. The conclusions are that some xenobiotics are adding to the unpredictability of and inability to perform risk assessments for genetically engineered vaccines. Furthermore, such vaccines should not be used within aquatic ecosystems until a number of pertinent questions have found satisfactory answers.

The final *chapter 7* asks for some changes of attitudes among scientists as well as politicians. Recent experiences ought to call for humility with regard to environmental effects of science and technology. In many cases "experts" were proven wrong after damage have been done. To the extent that any prior investigations of damaging effects had been undertaken, methods used were inadequate and only capable to reveal short-term effects, whereas the long-term impacts were the most important and serious.

At the present the definition of "safety" is very narrow in vaccinology. "Safety research" is occupied with prospects of unintended and unwanted side effects with regard to the targeted vaccinees themselves, or nontargeted individuals within the same species. This narrowing of conception and research strategies may leave potential hazards anapprised until they actually happen.

There is a most striking lack of holistic and ecologic thinking with regard to vaccine risks. This seems to be symptomatic for the real lack of touch between research in medicine and molecular biology on one hand, and potential ecologic and environment effects of these activities on the other.

In order to make reliable risk assessments and perform sensible risk management with regard to genetic engineering in general, and genetically engineered vaccines in particular, much pertinent knowledge is lacking. The prerequisite for obtaining such knowledge is science and scientists dedicated to relevant projects and research areas. It must be the responsibility of the national governments and international authorities to make funding available for such research. On one hand, this is obviously not the responsibility of producers and manufacturers. On the other hand, risk-associated research must be publicly funded in order to keep it totally independent, which is an absolute necessity for such activities.

Although vaccinology is the "Holy Grail" of medicine, there are other ways of preventing infectious diseases in humans and animals that must not be ignored. Many of the most burdening infectious agents of mankind and its domesticated animals are caused by pathogens that have reservoirs and are circulating among wildlife animals. By increasing our knowledge

about these reservoirs, their occurrence, the transmission routes within and out of the indigenous ecosystems, we might be able to break transmission chains, or keep our activities out of dangerous ecosystems. There is a void in knowledge about the ecological interactions for many important pathogens. This field is to some extent subdued by the confidence in vaccines, and hence it is another scientific orphan.

1 Introduction: the past and the present

1.1 Prevention or treatment?

The bicentennial celebration of the first vaccination took place just a few years ago. In 1796 Edward Jenner injected cowpox virus into the boy James Phipps, and later on challenged him with fully virulent human smallpox (variola) virus. The boy survived, and Jenner had hence protected him against one of the most dreaded human diseases of all times. The smallpox vaccination story ended in an triumphant eradication of variola virus, due to a world-wide vaccination campaign (Fenner et al., 1988). In recognition of Edward Jenner´s contribution, procedures which aim at protection against disease by pre-mobilization of the immune system were termed “Vaccination”, derived from the Latin word *vacca* In that context it is well worth for present day scientists to reflect on the fact that the contemporaries of Jenner rejected his findings. He had to be his own publisher, and publish at his own costs (Jenner, 1798). Peer review obviously would have refused him!

From an immunological standpoint, perhaps the most obvious strategy is to obtain protection against an illness by prior infection with a weaker or related version of the actual pathogen. The ancient Chinese protected against smallpox by *variolation*. Small quantities of scabs from an infected person were intranasally inoculated (Fenner et al., 1988). Edward Jenner used cowpox as the related immunogen against smallpox. By testing his procedure scientifically, he established the precedent for using a related but less dangerous pathogen to elicit immune responses that are cross-protective against the more virulent pathogen (Jenner, 1798; Baxby, 1999). The widespread use of other live vaccines against human and domestic animal infectious diseases is testament to the great success of attenuated viruses (Liu, 1998).

This procedure was first applied to bacterial pathogens by Louis Pasteur in experiments demonstrating protection of chickens from cholera and sheep from anthrax (Pasteur, 1881 and 1882). The most widely known attenuated bacterial vaccine is, however, bacille Calmette-Guerin (BCG) for protection against tuberculosis. It was first used in 1921 (Calmette, 1927) and is still used today.

Vaccination intends to provide the individual with immunological protection before infection takes place. If the vaccinated proportion of a population is high enough disease symptoms of the individual as well as transmission of the disease agent may be prevented. Ultimately this may result in eradication of the disease agent, as illustrated by the small pox vaccination campaign, which is now sought repeated for poliovirus (Bloom and Widdus, 1998).

The reasons for giving priority to prevention and prophylaxis instead of therapy are stronger than ever. The therapeutic opportunities offered by chemotherapeutics and antibiotics are reduced due to resistance developing in microorganisms, viruses and cancer cells. Furthermore,

for many infectious and neoplastic diseases damage done before symptoms of disease are recognized will result in lasting damaging effects, death or loss in production even if efficient therapy is available.

Vaccines and vaccination represent areas of huge economic interests. In 1993 the world market for vaccines was estimated at approximately US$ 3600 millions, split about equally between the human and veterinary sectors. The fish vaccine market made up an estimated US$ 12 millions, with about US$ 7 millions for Norway alone (Zänker and Vershueren, 1997).

Traditional vaccines have had great impacts on, and some of them have been immensely successful within, both medicine and veterinary medicine. For example, in the USA within a period of 5-10 years after the introduction of vaccines, polio, diphteria, neonatal tetanus, measles and rubella were all but eliminated (Folkers and Fauci, 1998). And childhood immunization is one of the most cost-effective medical interventions available. The US Centers for Disease Control (CDC) estimates that for every $1 invested in immunization, between $2 and $29 are saved. The entire cost of the Global Smallpox Eradication program, about $32 millions, is returned every 20 days in not having to vaccinate travelers (Bloom and Widdus, 1998). But the most valuable revenue of vaccines is of course the millions and millions of saved lives. Furthermore, the socioeconomic and human life-saving effects of vaccines used within veterinary medicine might hardly be over-estimated.

Through progress in molecular immunology and genetic engineering, opportunities to produce vaccines for a number of purposes and target organisms (mammals, birds, fish, plants) have been dramatically improved. The new strategies may give rise to vaccines which are pure, provide efficient and long-lasting immunological responses at a low price, and have decreased potentials for unpredicted side-effects in the vaccinated individuals. On the other hand theoretical, unpredicted harmful effects and hazards to the environment and specific ecosystems may be more than worst-case scenarios. In that perspective, it is highly disturbing to find that so little efforts are dedicated to risk-associated research, and that much of vaccine-relevant research are dominated by commercial interests.

1.2 Immunity and the immune systems

(lat. *immunis* = exempt)

The life of every organism is constantly threatened by other organisms, this is the nature of the living world. To face this, all species have evolved protective mechanisms, i.e. camouflage colors, production of poisons or extremely effective running muscles. Through their continual battle with microorganisms and viruses, vertebrates have evolved an elaborate set of protective measures that are collectively termed the *immune system.* The word "immune" implies freedom from a burden: an individual that is immune to a specific infecting agent will be left unharmed when infected by that agent again.

The immune systems of multi-cellular organisms are composed of both *innate, nonspecific* and *adaptive, specific* components and mechanisms. The innate system may be rapidly activated to keep out or remove all macromolecules, infectious agents and cells that are «non-self». This defense system is composed of cells, i.e. macrophages, which internalize and digest intruders when they are present in body fluids, blood and tissues. Other effector cells, i.e. NK (natural

killer) cells are seeking up and killing cells within the body which contain microorganisms, viruses or «new» components, i.e. cancer cells.

The *adaptive, specific* immune system works by a learning process. The first encounter with a bacterial, fungal, protozoan or viral pathogen leads to an infection, which is often accompanied by disease symptoms and may even kill the individual. The immune system aids in recovery from the infection. Furthermore, after recovery the individual may remain free of the particular disease for ever. Through the first encounter the immune system has learned to recognize this specific pathogen as foreign, "non-self". Should it attack again it may be effectively kept out of the organism, or killed.

A key function carried out by the immune system is *recognition*. The system must recognize the presence of an invader. But it is equally important that it is able to discriminate between foreign invaders and the natural constituents of the body, i.e. between *non-self and self*. The importance of this is underscored by disorders, *autoimmune diseases*, in which such discrimination fails.

Recognition of non-self is the first step of an immune defense mobilization. It must be followed up by steps intended to eliminate the invader. The immune system thus carries out two sets of activities: *recognition processes* directed against individual discrete aspects of a target, and *destructive processes* that follow from recognition and allow the immune system to mount an attack against the invader

The specific immune system is composed of different cell populations which each is able to recognize and react towards only one particular non-self molecule. Hence, defense and attack mechanisms directed against and adapted according to, the particular infectious agent threatening the organism at the moment, may be mobilized. The first time an individual is exposed to a given agent (primary infection) mobilization of the adaptive immune system may take up to a couple of weeks. Hence, the symptoms may be lighter, but infection is not prevented. Following primary infection, «memory»-cells which have been educated to react rapidly to re-exposition remain in the organism. Upon a re-infection efficient immune reactions may hence be able to stop infection at an early stage. The strategy of vaccination is hence to carry out primary immunization before the individual becomes naturally infected.

Cells which make up the adaptive immune system are organized into two armies with different weapons and strategies for combat plus a corps of commanding officers which conduct, coordinate and modulate the activities. One army is composed of so-called B-lymphocytes, the other of cytotoxic T-lymphocytes (CTL). Helper T-lymphocytes (T_H) act as commanding officers.

When B-lymphocytes encounter the infectious agent they are pre-programmed to recognize, cell-division and maturation into plasma-cells is initiated. The antibodies are special proteins which may bind to and neutralize their corresponding infectious agent by various molecular mechanisms.

CTLs bind to the surface of body cells which are infected with the agent they are directed against and secrets substances (i.e. perhorins) which damage the membrane integrity of target cells or force them to commit suicide (programmed cell death, apoptosis).

Helper T-lymphocytes, upon contact with their pre-programmed target-agent will initiate production of factors (cytokines) which stimulate proliferation and activity of B-lymphocytes and CTLs that share their targets.

The mechanisms by which the immune system controls disease include the induction of neutralizing antibodies and the generation of T-cell responses, including T_H cells and CTLs. For some viruses and bacteria antibodies provide protection by preventing the virus from entering cells or by recruiting bactericidal mechanisms and neutralizing bacterial toxins. T_H cells also contribute to resistance against viral and bacterial infections by producing cytokines and other bioactive molecules that cause inflammation and stimulate antibody, macrophage, and CTL responses. Traditional vaccines stimulate antibody, and to varying extents, T_H cell responses, and thereby protection against some infections.

In contrast, antibody and TH cell responses do not eliminate most cancer cells and many viral infections. In such situations protection may be provided by CTLs, which kill diseased cells. But, as earlier stated, most conventional vaccines, which are composed of inactivated infectious agents or their subunits, fail to elicit CTL responses. This has been a major limitation in the development of immunotherapies against viral diseases and cancer. The present review will deal with some of the approaches to circumvent these limitations.

Antigen display and presentation

In order to stimulate T lymphocyte responses, peptide fragments from foreign antigens and infectious agents must first be bound to peptide binding receptors, major histoccompatibility complex (MHC) class I and II molecules, in order to be displayed to the immune system on the surface of professional antigen presenting cells (APCs). The professional APCs, such as dendritic cells and macrophages, shuttle to lymphoid organs, which are the locations where immune responses are initiated. There the APCs present antigenic peptides very efficiently and deliver all required activation signals to T cells. T lympocytes produce an antigen receptor that they use to monitor the surface of APCs for the presence of foreign peptides. The antigen receptors on T_H cells recognize antigenic peptides bound to MHC class II molecules whereas the receptors on CTLs react with antigens displayed on class I molecules. In addition to recognizing foreign antigens, T cells often need additional stimulation to become fully activated. These additional signals are delivered through other receptors (e.g. CD28 and CD40L) on the T cells. These react with ligands (e.g. B7 and CD40) that are present on professional APCs but are absent from most other cell types.

Mucosal immunity

Most human and domestic animal pathogens initiate infection at mucosal surfaces, where they encounter the body's first and most effective line of defense: the mucosal immune system (MIS), which is in many ways distinct from the systemic immune system. MIS constitutes an integral part of mucosal surfaces, e.g. the linings of the gastrointestinal, respiratory and urogenital tracts (Lamm, 1997). The best studied mucosal tissue is the gastrointestinal tract, where the Peyer patches (PP) are found. The epithelium of PP are enriched with antigen sampling cells known as M cells. These cells transport antigens, including whole microorganisms, from the intestinal lumen to the follicle underneath, where B and T cell responses are induced (Jepson and Clark, 1998). Mucosal immunity is characterized by secretory immunoglobulin A antibodies (IgA). Activated lymphocytes leave the PP via the

lymphatic system and enter the systemic circulation, moving to other places in the body and homing to other mucosal and glandular tissues. Sensitizing one part of the MIS will stimulate other parts, but it has become clear that mucosal immunization induces stronger responses at, or adjacent to, the site of induction than at distant places. Furthermore, portions of the MIS, especially the urogenital tract, seem to function more independently than others (Mestecky et al., 1997). Ideally, vaccines against pathogens that cause, for example respiratory and diarrheal diseases should sensitize and mobilize the MIS.

Memory

The immune response generated during primary infection is such that when there is a secondary encounter with the pathogen the host is better able to prevent disease. This is due to what is termed "immunological memory". The cells responsible for this activity are antigen-educated T and B lymphocytes that can persist for long periods of time and are capable of reactivation following an appropriate reencounter with antigen (reviewed by Campos, 1998). Primary B- and T-cell responses are different from those generated by antigenic rechallenge. The functional behavior of memory cells, however, develops in response to antigen-specific and –nonspecific signals received during the primary encounter with the antigen. Thus, memory cells are educated during the primary response.

Immune memory mechanisms are recognized by their potential to mount an enhanced secondary immune response, often a long time after primary immunization. *This phenomenon is the foundation of vaccination*, and the cellular and molecular mechanisms behind it are starting to be elucidated (reviews by Gray, 1993; Campos 1998).

It is generally accepted that secondary effector cell populations are derived from a specific subset of T and B cells that develop during primary immune responses. Compared to naïve cells, memory cells have a faster response time, specialized tissue localization and more effective antigen recognition and effector functions.

Memory cell proliferation depends on a number of factors, including antigen recognition, costimulatory signals, and activation by exogenous molecules such as cytokines, hormones and neuropeptides.

It should be stressed that induction of memory cells does not assure protection from a second challenge infection. It is hence essential to discriminate between immunological memory as a biological entity and the complex reactions and mechanisms of protection from disease upon secondary infection (Doherty, 1994).

Cytokines

Cytokines are signal peptides produced, in principle, by one type of immuno-responsive cell to instruct another about actions to be taken. There are various groups and types of cytokines. Some of the best known cytokines fall within the groups interferons (IFN), interleukins (IL), tumor necrosis factors (TNF) and tumor growth factors (TGF). They act by binding as ligands to specific receptors on responsive cells. The ligand-receptor reaction initiates intracellular signal transduction pathways ending up in the nucleus where the expression of a set of cellular genes is up- or downregulated. The net result obtained may be cell differentiation, or that sets of cells are promoted to take specific actions.

It is now clear that cytokines regulate both the initiation and maintenance of the immune response. Moreover, they select the type of immune response and the effector mechanisms that mediate resistance to pathogens. However, certain cytokines, particularly when produced in excess, can induce pathogenesis (reviewed by Fresno et al., 1997).

The TH1/TH2 paradigm

Considerable evidence has accumulated to suggest the existence of functionally different subsets of THs and CTLs (reviewed by Romagnani, 1997). TH1 cells produce IFN-gamma and TNF-alpha. These cytokines activate APCs, and are also involved in delayed-type hypersensitivity reactions. By contrast, TH2 cells produce IL-4, IL-10 and IL-13. These cytokines are responsible for strong antibody responses, and inhibit several macrophage functions. T cells expressing cytokines of both patterns have been designated TH0 (Romagnani, 1996).

TH1 and TH2 cells do not seem to derive from distinct precursors, but rather develop from a common precursor under the influence of environmental and genetic factors acting at the level of antigen presentation. Among environmental factors the route of antigen entry, the physical form of antigen, the type of adjuvant and the dose of antigen seem to be of significance (Constant and Bottomly, 1997). The genetic factors are yet unidentified.

The environmental and genetic factors bear impacts on the TH1/TH2 differentiation mainly by determining which cytokines are present in the environment of the responding TH cell. Presence of IL-4 is the most potent stimulus for TH2 differentiation, whereas IL-12 and IFNs favor TH2 development (Seder and Paul, 1994). Recently it also became clear that IL-6 derived from APCs is able to differentiate naïve TH cells into effector TH2 cells by inducing the initial production of IL-4 (Rincon et al., 1997).

TH1 responses are most important for protection against intracellular pathogens, e.g. viruses and some bacteria and protozoa. TH0 responses take better care of extracellular pathogens, and TH2 responses give optimal protection against metazoan parasites (Daugelat and Kauffman, 1996; Romagnani, 1997).

If activation of the adequate TH subset fails, a given immune response may be inefficient. On the other hand over-activation of certain subsets, or a sub-optimal balance between the subsets may result in pathological injury to the host organism (reviewed by Romagnani, 1997).

Conformational epitopes

T cells recognize small linear peptides in the context of MHC molecules. Antibodies that neutralize viruses, however, generally recognize antigenic determinants that are created by the three-dimensional conformation of the protein or glycoprotein antigens on the pathogen. Hence, isolated proteins from pathogens may evoke immune responses, but fail to elicit neutralizing antibodies essential for protection against the actual pathogen.

Genetic and antigenic variation among pathogens

For a number of important pathogens closely related, but antigenically somewhat different, strains are found. Other pathogens undergo rapid mutation and selection of antigenic mutants when made subjects to immune attacks. Antibodies, and to a lesser extent also T cell responses, are often restricted to the initial immunizing strain or variant. In some cases these immune

responses will not protect the host against disease upon later encounters with other strains or variants of the particular pathogen. In some cases preimmunized individuals are even more prone to disease upon repeated exposures. This may be due to the phenomenon termed *antibody-dependent enhancement of infection.* It implies that when an individual with antibodies against a given virus becomes reinfected with a slightly different viral variant or strain, the preexisting antibodies will react with the new virus without neutralizing it. Such un-neutralized virus-antibody complexes may be taken up by cells that have cell-surface receptors for antibodies, e.g monocytes or macrophages, and initiate very efficient infection in them. As illustrated by Dengue haemorhagic fever, symptoms in reinfected individuals may be much more serious than in the primary infected. By the same mechanism antigenic variation may represent an obstacle for, and potential hazard related to, vaccination.

1.3 Vaccines and vaccination

All vaccines have in common the intention to prevent disease or limit the effects of disease. Both humoral (antibody-mediated) and cellular arms of the immune system can contribute to a pathogen-specific acquired response that distinguishes specific immune protection from the innate and more general protection mediated by phagocytes (i.e. macrophages and dendritic cells), cytokines and physical barriers. Because vaccination against a threatening disease may take place many years before exposure to the pathogen, immunological memory is a critical element. A long-lived immune response which may be mobilized and augmented rapidly when called for, is essential.

Vaccination may have different purposes and fields of application. The most important are

- Protection and treatment against infectious diseases
- Protection and treatment against cancer
- Induced infertility in humans, domestic animals and wildlife

An ideal vaccine provides an optimal mobilization of the adaptive immune system with no unwanted side effects, and with long-lasting immunological memory.

The most universal purpose is to prevent disease in individuals and prohibit transmission of disease agents between individuals. Generally, vaccination must be carried out before the individual becomes infected, but for some diseases, i.e. rabies, disease may be prevented even if vaccination take place after infection.

Some important human and domestic animal pathogens, i.e. rabies virus, hantaviruses and a number of arboviruses, have reservoirs in free-ranging wildlife animals. Human and animal disease may then be prevented by vaccination of reservoir animals.

Likewise, some free-ranging mammalian species are considered «pests» in the context of human food, animal fodder or other kinds of production. Enforced infertility following vaccination is now becoming an alternative to culling (“stamping out”) for control and reduction of such pest animal populations.

Cancer cells often express surface antigens not present on their normal counterparts. Such unique antigens may provide targets for vaccines which may induce immune reactions to prevent and combat cancer cells.

Depending on the species, target-organs, epidemiological considerations etc. the delivery method and route may differ. In practical terms, the vaccine may be delivered by:

- Injection, most commonly intramuscularly or subcutaneously. In a recent further development of injection, so-called "gene guns" are used to propel small gold particles covered with antigen through the skin. Such procedures are often referred to as "biolistics".
- Inhalation of vaccine-containing aerosols.
- Ingestion of vaccine-containing vehicles, i.e. capsules.
- For fish: Bathing in or spraying with vaccine containing solutions

For vaccination of free-ranging animals the vaccine is usually offered in baits which are spread out over the selected target area from airplanes or helicopters.
For ingestion a new concept is now coming up, namely vaccines produced by so-called «bioreactors», transgenic animals or plants. For instance, a genetically modified (GM) crop plant is expressing an inserted gene which codes for a protein originating in an infectious agent or a cancer cell. Following consumption and digestion in the gastro-intestinal tract the vaccine protein is released and initiates a specific immune reaction. Similarly, for transgenic animals the vaccine protein may be secreted in for instance milk.

To a varying extents, all the vaccine delivery strategies imply that vaccine-containing materials may end up in unintended locations, and hence release or escape of biologically active substances (i.e. DNA or RNA), viruses or microorganisms may take place.

1.4 Traditional vaccines

Until recently most vaccines were of the «whole disease agent»-type: the whole bacterial cell or virus particle was used for immunization after varying degrees of purification. Such vaccines might be killed, inactivated or «live». Live vaccine agents (i.e. bacteria or viruses) are able to infect the vaccinees, but have had their disease-provoking abilities and/or host-cell preferences attenuated through passage in unnatural host organisms or cells.

The first generations of vaccines were based on viruses and microorganisms propagated in laboratory animals (review by Hilleman, 1998). They were used after having been chemically inactivated. Such vaccines had to be injected, often more than once. They often gave adverse immunological reactions due to impurities. They gave no local immunity on the epithelial surfaces of the body, which are the portals of entrance for most infectious agents. They generally resulted in satisfactory antibody production (humoral response), but inadequate cellular immune responses.

Modern era viral vaccinology began in 1949 with Enders' cell culture breakthrough that made possible a series of live, attenuated and killed virus vaccines. Live vaccines gave a stronger mobilization of all effector parts of the immune system, and might also, if properly delivered, give good local immunity on the epithelial surfaces which come into initial contact with

invading viruses and microorganisms. The most prominent draw-back of live vaccines was reversion to full virulence, which took place sometimes and posed a potential risk to the vaccinee, but also to his/her contacts.

The subunit hepatitis B vaccine represented a new strategic breakthrough in 1981. «Subunit» means that the part(s) of the disease agent which is the basic for eliciting protective immune responses has been separated from the virus/microorganism itself, and purified by modern separation technology. This in principle may give less unwanted immune reactions.

Finally, the first recombinant expressed vaccine, also against hepatitis B, was licensed in 1986. In reality this is also a subunit vaccine, but the polypeptides from HBV (hepatitis B virus) has been expressed from genetically modified yeast.

Newer technologies (see below) have not led to licensed products. «We are still tied to the past» (Hilleman, 1998).

1.5 Vaccine delivery

During the last years there has been considerable progress in techniques to identify antigens that are important for immune system mobilization against various infectious agents and cancers. But methods for delivering these antigens are, in many cases, crude and inadequate.

Immunization with antigens alone often elicits weak or no immunity, and the responses may be restricted to antibodies and TH cells. Such responses do not eliminate most cancer or virally infected cells. In such situations protection is provided by CTLs, which kill the diseased cells. Most conventional vaccines, which are composed of inactivated pathogens or their subunits fail to elicit CTL responses, and this has been a major limitation in the development of immunotherapies against infectious diseases and cancer. Some of the reasons for the lack of CTL stimulation by conventional vaccines have been eludidated and a number of strategies are being pursued to circumvent these limitations.

More adequate immune responses may be elicited if antigens are administered in combination with *adjuvants*, which are immunostimulating agents. However, many adjuvants produce undesirable side effects, such as severe inflammation, which has precluded use in humans or domestic animals. The only adjuvant that is currently approved for use in man is alum (aluminium hydroxide gel), which is a relatively weak potentiator of immune responses. Most adjuvants that have been tested support the generation of some kinds of immune responses, but fail to mobilize other important arms of the immune response such as cytotoxic T lymphocytes. The mechanisms of action of traditional adjuvants are poorly understood (Raychaudhuri and Rock, 1998). They are thought to potentiate immune responses by several means. These include inducing inflammatory processes, which upregulate or stimulate de novo production of key molecules (e.g. cytokines, adhesion, and costimulatory proteins) that are necessary for the generation and amplification of immune responses. In addition, several adjuvants provide a depot of antigen that is thought to sustain the stimulation of immune responses and may stimulate APCs to acquire more antigen. Because the antigen depot is extracellular, it is presented exclusively on MHC class II molecules. Consequently, these kinds of adjuvants stimulate T_H cell dependent responses, but do not generally induce CTL responses. Hence a

number of strategies are being pursued to enable vaccine antigen presentation on class I molecules and thereby elicit CTL immunity.

A number of new adjuvants that elicit CTL responses in some cases have been developed lately. Some of these adjuvants, like ISCOMs, QS21 and AF seem to work by facilitating delivery of antigens into the cytoplasm of cells, to be processed and presented as antigenic peptides on MHC class I molecules.

One approach to elicit CTL responses is to introduce genes coding for antigens into the host so that APCs will synthesize the antigen themselves, and therefore present antigenic peptides on MHC class I molecules. *Genetically engineered viral or bacterial vectors* that invade the cytoplasm of cells are now being used to introduce antigens into APCs. Vectors that are being exploited include poxviruses, adenoviruses, herpesviruses, baculoviruses, Listeria monocytogenes, Salmonella and Mycobacterium bovis bacillus Calmette-Guerin (BCG) (see section 2.4). These viruses and organisms have potentials to cause disease in humans and animals. Safety issues must therefore be thoroughly addressed before such delivery systems may gain widespread acceptance. An additional problem with this delivery strategy is the production of neutralizing antibody responses to the vector, which may render further immunizations ineffective. An alternative method is to inject *genetically engineered plasmid expression vectors* with inserted antigen genes so that they are internalized, trancribed and translated by host cells (see section 2.5). The use of *genetically engineered RNA* as vaccine delivery system for the induction of antigen-specific immunity is also coming up (see section 2.6). In principle, these approaches allow repeated immunizations to be effective. But there are two inherent problems with these approaches: plasmids may integrate into host cell chromosomes and anti-nucleic acid immune responses may be elicited.

Complex antigen preparations that physically mimic viral particles (*virus-like particles, VLPs*) have been found to elicit CTL responses. VLPs containing antigens from HIV, other viruses, and multiple malaria epitopes have been produced (review in Raychaudhuri and Rock, 1998).

One approach that seems to satisfy most criteria for vaccine development is the use of *particulate antigen delivery systems* to introduce antigens into endosomes or cytoplasma of cells, and hence to become presented by MHC class I or class II molecules. CTL responses have been achieved by immunizing animals with exogenous antigens conjugated to small latex, iron or PLG (poly-lactide-co-glyolide) particles or spheres. PLG particles seem particularly promising, since it has a long history as sutur material in humans and domestic animals, while no side effects have ever been recorded (Raychaudhuri and Rock, 1998).

Immunoregulatory molecules as adjuvants

The cloning and characterization of many immunoregulatory molecules, e.g. cytokines, have opened perspectives to manipulate immune responses with biologically active molecules. Such molecules might be used as well-defined *molecular adjuvants* to generate stronger immunity or to alter the kind of immune responses (review in Raychaudhuri and Rock, 1998).

Some factors, such as GM-CSF (granulocyte-macrophage colony-stimulating factor), Flt3L, IFN-_ (interferon-_), and IL-12 (interleukin-12) stimulate the propagation or activity of APCs. Others, such as B7, IL-2, and IL-15 stimulate the activation and growth of T lymphocytes. Still others, as IL-4, IFN-_ and IL-12 can alter the menu of cytokines produced by T lymphocytes

and consequently also their function. They may for instance force T cells to differentiate into IFN-_-producing TH_1 cells or IL-4-producing regulatory TH_2 cells. This is important because in some cases TH_1 responses are protective whereas TH_2 responses exacerbate disease. In principle, these factors could be combined with any antigen delivery system to improve immune responses qualitatively or quantitatively , but they will not allow antigen preparations to elicit CTL responses if they are not presented on MHC class I molecules.

Vaccine-based therapy

Some investigations, trials and data exist which indicate that vaccination can be used not only to prevent, but also to treat infectious diseases. Animal trials and limited clinical trials have in some cases demonstrated dramatic, positive changes in symptoms and courses of illness (e.g. Nesburn *et al*., 1994, Benton and Kennedy, 1998). Steps are now being taken to clarify the therapeutic effect of such superimmunisation. A number of major, controlled trials are currently being performed involving vaccination of people with herpes infections, leprosy, tuberculosis, leishmaniasis and hepatitis B (Cohen, 1993). If a positive effect can be documented for these, and perhaps also for other infectious diseases, the total need for, and use of, effective vaccines will rise greatly. In that case, this tendency will be fortified by the need for alternatives to antibiotic treatment since the problem of resistant bacteria will only become more serious, and effective therapeutics against viral infections and cancers will still not exist.

2 Genetically engineered vaccines: Strategies and alternatives

Modern molecular biology, recombinant DNA technology and genetic engineering have opened the road to a number of alternative strategies for vaccine production.

2.1 Subunit vaccines

Subunit vaccines represent technologies ranging from the chemical purification of components of the pathogen grown in vitro to the use of recombinant DNA techniques to produce a single viral or bacterial protein, such as hepatitis B surface antigen for example.

If the DNA sequence coding for the immunogen(s) of a given infectious agent, cancer cell type etc. is known, the sequence can be translated into aminoacid sequences, and corresponding peptides may then be synthesized in the laboratory. The peptides may, when they are injected or spray on mucous membranes, elicit protective immune responses directed against the infectious agent/cancer cell. A disadvantage of this approach is that immune responses, especially T-lymphocyte activation, have been too weak. In addition, production is relatively expensive. But since quite pure immunogens may rapidly be produced in large quantities, it is evident that this strategy has a great future potential and intensive research and developmental activity take place within this field.

2.2 Recombinant vaccines

Recombinant DNA is made by isolation of DNA fragment(s) coding for the immunogen(s) of an infectious agent/cancer cell, followed by the insertion of the fragment(s) into vector DNA molecules (i.e. plasmids or viruses) which can replicate and conduct protein-expression within bacterial, yeast, insect or mammalian cells. The immunogen(s) may then be completely purified by modern separation techniques. In cases where essential antigenic epitopes are conformationally determined this approach may give better results than corresponding synthetic vaccines, but otherwise the theoretical drawbacks are the same: the vaccines tend to give good antibody responses, but weak T-cell activation. A number of recombinant vaccines have already been marketed.

2.3 Genetically modified microorganisms and viruses as homologous vaccines

Especially for viruses, but recently also for some bacteria, «disease-causing» genes have been identified and characterized. For some viruses it has been possible to genetically engineer, i.e. by introducing deletions or specific point mutations, without destroying the ability of the virus to infect its target host species by a natural route. When such a genetically modified virus is used as vaccine, the immune system will be stimulated and activated in the same way as by a natural infection, but with no or substantially weaker, symptoms. Such vaccination may result in very efficient and long-lasting protection since it results in optimal stimulation of both B- and T-lymphocytes. In addition local immunity on the epithelial surfaces used as portal of entries during natural infection may be detained.

2.4 Live vector vaccines

The most commonly used strategy is to insert the DNA fragment(s) coding for an immunogen(s) intended for vaccination into the genome of a «non-dangerous» virus or bacterium, the vector.

The insertion is performed in such a way that the vector is still infectious («live»). When such a recombinant vector-virus infects an individual, the inserted vaccine-gene as well as the virus' own genes will be expressed, and the gene products (proteins) will be present within the infected host cells. This may result in a highly efficient activation of both B- and T-lymphocytes directed against the immunogen and hence the infections agent or cancer cell from which the inserted gene originates. In addition important local immunity may be evoked if the vector is able to infect the epithelial surface that the infectious agent in question is regularly using as its portal of entrance. Some vectors i.e. bacteria and viruses within the family Poxviridae, permits insertion and expression of genes (at the cDNA level) from a number of infectious agents without loosing its infectivity. This makes it possible to immunize against a number of infectious diseases with one vaccine shot, aerosol-inhalation or capsule-ingestion.

2.4.1. Viruses

Poxviruses
Vaccinia virus and other orthopoxviruses have been extensively evaluated and used as live genetically modified vectors for vaccination against contagious diseases and cancers, recently also against fertility in pest animals. Orthopoxviruses are able to undergo genetic recombination, both within and across species borders, which may lead to hybrid progenies.

Vaccinia virus (VV)
The virus belongs to the Orthopoxvirus genus within the family *Poxviridae*. This is a large family with known representatives among all vertebrates and a number of insects. It is safe to assume that a large number of members, also orthopoxviruses, are still unknown. Unmodified VV as a vaccine was the central component of the successful eradication campaign against the dreaded smallpox disease. The origin of VV is uncertain, but it may have developed by repeated recombinations between different orthopoxviruses.

VV has a very broad spectrum of susceptible host animals, making spread within ecosystems across species borders a real possibility (Moss, 1996; Fenner, 1996).

VV as vaccine vector

VV has an approximately 180 kbp DNA genome. Parts of the genome may be removed by recombinant DNA techniques (gene technology) and replaced by some foreign DNA, without destroying the ability of VV to infect host cells. VV with foreign gene inserts up to approx. 30 kbp will infect inoculated individuals, replicate and express the proteins that the foreign gene(s) are coding for. The inoculated individual will then mount an immune response against that foreign gene product. If the foreign gene is taken from another virus (i.e. rabies virus), this immunity may protect, partly or totally, against a later natural infection.

VV-based recombinant vaccines consist of live, infectious virus which is shed from vaccinated individuals. Human beings or domestic animals will not be placed in isolation or guarantine after vaccination. Consequently, not only wild life vaccinations, but all practical applications of VV-based vaccines automatically means release of genetically engineered virus.

VV has a number of important theoretical and practical advantages as a gene-vector: both humoral and cellular immunity are elicited; its large genome render multivalent vaccines feasible; the entire life cycle takes place in the cytoplasm, minimizing risk of integration; poxvirus promoters are not recognized by the eukaryotic trancription machinery, and vice versa; the virus DNA is noninfectious; vaccines are easy to produce; the virions are very resistant, i.e. there is no need for refrigeration. It is easy to conceive that at least one of the listed advantages - stable and resistant virions (virus particles) (Pastoret et al., 1996) - may represent a serious draw-back in an environmental connection.

There is also a number of disadvantages by using recombinant VV: Unmodified VV may cause serious, generalized infections in some individuals with immunosuppressive disorders or treatment schemes; the potential host spectrum of VV is very broad, besides laboratory animals pigs, cattle, camel and monkey species are susceptible, but the list is certainly not complete; there is a high number of closely related viral species, and high degrees of sequence homology across species borders; VV has a large potential for engaging in recombinations; and VV particles have a high degree of environmental resistance, rendering spread and transmission possible by insects, migratory birds and animals, domestic and pet animal trade etc. A number of these disadvantages are theoretical, having modest scientific support, mainly because research which might prove or disapprove them have never been carried out.

In order to vaccinate reservoir animals in Europe (red foxes) and North-America (racoons) recombinant VV/rabies vaccine has been released over vast land areas (Brochiv et al., 1990; Anderson et al., 1991; Pastoret et al., 1996)

The rabies vaccine is but the first of a series of poxvirus-based genetically engineered viruses that we will be confronted with in the future. A number of these, for medical and veterinary purposes, are at various stages towards approval in USA, EU etc.

Avipoxviruses as vectors

Avian poxviruses offer an alternative to VV vectors. These viruses possess many of the desirable characteristics of VV (Baxby and Paoletti, 1992). They readily infect mammalian

cells *in vitro* and induce synthesis of foreign gene products. Despite this, avipoxviruses are claimed to undergo abortive replication in non-avian cells and cannot be adapted to produce infective progeny in mammalian tissue (Fries et al., 1996), although the experimental data (Taylor and Paoletti, 1988; Taylor et al., 1988) for such a definite conclusion seem meager. Despite their inability to replicate in mammalian hosts, recombinant avipoxvirus vaccines have induced protective immune responses to their inserted foreign gene products in several mammalian species (Taylor et al., 1991; Fries et al., 1996).

A canarypox virus recombinant based on an existing attenuated veterinary vaccine and carrying the gene for rabies virus glycoprotein (ALVAC-RG) has been shown to induce neutralizing humoral responses and protective immunity in mice, cats and dogs (Taylor et al., 1991). A study in human volunteers reached the conclusions that this vaccine was safe and induced both functional antibodies and cellular immunity against rabies virus (Fries et al., 1996). Various other avipoxvirus vector vaccines are in clinical trials for the moment.

Adenoviruses

Adenoviruses can efficiently induce immune responses in the lung following single gut delivery. These viruses can also be genetically engineered to express a number of heterologous proteins *in vitro,* and in the past 10 years, recombinant adenoviruses expressing a variety of antigens have been constructed and tested (reviewed by Imler, 1995).

Engineered adenovirus vectors offer a number of interesting features to develop new vaccines: they are supposed to be associated with only benign pathologies in humans; their genome has been extensively studied and the complete DNA sequence is known for some serotypes; methods to construct recombinant vectors are well established; and they can be administered orally. Furthermore, it has been claimed that the transferred genetic information remains epi-chromosomal, hence avoiding insertional mutagenesis and alteration of the cellular genome (Graham and Prevec, 1992). Several studies have been published describing immunization of different animal species with various adenovirus vectors (review by Imler, 1995). For instance, mice were vaccinated with a replication-defective recombinant adenovirus expressing a single malaria antigen, the CS protein (Rodrigues et al., 1998). Protective immunity with a similar or even higher efficiency to that induced by radiation-attenuated sporozoites was induced.

Adenoviruses have a ds DNA genome of about 35 kbp. The genome is packaged in an icosahedral protein capsid with a 70 nm diameter. The adenoviruses which are mainly used as vaccine vectors replicate to high titers in cell cultures. It is of particular interest that, contrary to many other potential vector viruses host cell division is not required for infection and viral replication. The genome is divided in early (E) and late (L) regions, respectively, expressed before or after replication of the viral genome.

Recombinant adenoviruses can be constructed either by insertion or replacement of viral sequences. Depending on the chosen strategy, the resulting vectors are replication-competent or defective. The size of DNA that can be packaged in the capsids represents a limit to the insertion of foreign genetic material in adenovirus vectors. The size of the total recombinant genome must not exceed 105% of the unmodified viral genome (Bett et al, 1993). The principal sites of insertion or replacement of viral sequences with exogenous DNA are the early regions E1, E3 and E4 (Imler, 1995).

The popularity of adenoviruses as recombinant viral vectors is largely due to the successful and, seemingly, safe oral immunization of millions of US military recruits with Adenovirus 4 and 7 for prevention of respiratory disease outbreaks (reviewed in Grunhaus and Horwitz, 1992). Protective antiviral immunity was obtained in the airways after installation in the gut. The mechanisms of this phenomenon is unclear, but they might involve the general mucosal immune system. However, there might also be a direct effect on the airway mucosae, since adenovirus excretion in the pharynx could be demonstrated following enteric inoculation (Schwartz et al., 1974).

Following these first trials, a number of recombinant adenoviruses, intended for vaccination of hunmans and domestic animals, have been constructed and tested in animals (reviewed by Imler, 1995). They have been administered by various routes: intranasal; subcutaneous; intraperitoneal; or intratracheal. Most protocols induced an immune response, irrespective of the antigen, animal model or route of administration, and protection against challenge has been demonstrated in a number of cases.

For obvious safety reasons, it would be preferable to work with replication-defective recombinant adenoviruses. However, most of the studies performed in animal models involved the use of replication-competent viruses (reviewed by Imler, 1995).

Based on encouraging results obtained in animals, human clinical trials with a recombinant adenovirus 7 expressing the HbsAg were initiated. The adenovirus-HbsAg recombinant vaccine virus was coated on enteric capsules and given orally to volunteers. No anti-HbsAg antibodies were induced (Tacket et al., 1992).

Herpesviruses

Recently, considerable efforts have been made to develop herpesvirus vectors for vaccine delivery. Such vectors have been designed to accommodate entire genes, as well as parts of genes encoding a protective epitope (reviewed by Sheppard and Boursnell, 1998).

Herpesviruses have a relatively large genome with several genes being identified as "nonessential" both in vitro and in vivo. This offers a variety of potential sites for insertion of foreign DNA, and hence insertion of more than one gene into the same vector genome. This might allow a single vaccine for multiple pathogens or to vaccinate against the same pathogen with multiple antigens,

A limited number of herpesvirus vector-based vaccines have been tested with success in natural hosts. For instance, pigs have been protected against hog cholera infection by vaccination with an attenuated pseudorabies based vector expressing the E1 gene of hog cholera virus.

Poliovirus and other picornaviruses

Live vaccines against poliomyelitis, which have proved extremely safe and effective, are based on attenuated variants of the virus itself. Efforts have therefore been made to exploit such viruses as vaccine vectors. It is, however, not possible to insert more than a few foreign amino

acids into the poliovirus capsid. But small peptides corresponding to immunogenic epitopes may be expressed, and have in some cases elicited immune responses (reviewed by Sheppard and Boursnell, 1998).

Alternative picornaviruses have also been investigated as potential recombinant vectors, i.e. Mengo virus and various rhinoviruses.

Other positive-stranded RNA viruses
Alpha viruses are positive-stranded RNA viruses that are used as vector systems to deliver the genes of heterologous pathogens (reviewed by Liu, 1998). These viruses are attractive because they make many copies of the mRNA that encodes the structural proteins of the virus. This amplification of the mRNA has the potential to rapidly produce increased quantities of antigen. Replicons can be engineered to consist of the viral coat containing the genome in which the sequences for the viral structural genes have been replaced by the sequence encoding the selected antigen. Following infection of host cells the alphavirus can no longer replicate because it no longer contains the sequences for the necessary structural proteins. But large quantities of RNA encoding the antigen of choice, and hence large quantities of the antigen are produced (Frolov et al., 1996; Xiong et al., 1989).

One particular alphavirus, Venezuelan encephalitis virus, has tropism for the professional APCs, follicular dendritic cells in the lymph nodes. This virus is thus of particular interest as a vaccine vector for genes from some specific pathogens (Davis et al., 1996; Caley et al., 1997).

Influenza virus
Recently developed methods have made it possible to genetically manipulate influenza viruses. A possible advantage of influeza virus as a vector is the fact that repeated immunization might be possible without problems with immunity to the vector itself Sheppard and Boursnell, 1998). This is on account of the opportunity to vary the most important antigens of the actual influenza virus itself. Furthermore, cold-adapted influenza viruses have been extensively used as safe vaccines, and these might form the basis of recombinant vaccine vectors.

2.4.2. Bacteria

In the search for improved delivery of vaccines, much recent attention has focused on the use of several bacterial species which may lend themselves to the delivery of a variety of antigens from many different infectious agents. Among these are *Salmonella* species; BCG (bacille Calmette Guérin; *Streptococcus* and *Staphylococcus* species; *Listeria monocytogenes*; and enteric bactera as *E. coli*, *Vibrio cholerae*, *Yersinia enterocolitica* and *Shigella flexneri* (reviewed by Jones, 1998).

As recombinant vaccine vectors, any of these organisms may be delivered parenterally. But most importantly, by virtue of their ability to colonize or infect mucosal surfaces, most of them lend themselves to delivery to these surfaces, potentially evoking mucosal immune responses. Furthermore, these organisms have the potential to be used for the expression of multiple antigens. This make them important in the continuous search for new methods of developing combined vaccines (reviewed by Jones, 1998).

Attenuation of *Salmonella* spp, *E. coli* and BCG has allowed these human pathogens to colonize or invade the host with a minimum of adverse reactions. Attempts to attenuate *Vibrio cholerae* and *Yersinia enterocolitica*, on the other hand, have met with only mediocre success. Recently, attenuated and genetically engineered *Salmonella typhimurium* has been extensively exploited as a potential vaccine vector for immunization against various parasitic diseases of the Third World countries. It has been difficult to design vaccines against such disease agents, mainly due to their abilities to take on immunological disguise, or depress immune reactions. In mouse models immunity, sometimes protective, have been obtained with recombinant Salmonella carrying genes from such parasites. This concerns, among others, *Onchocerca volvulus* which causes human onchocerciasis or "river blindness" (Catmull et al., 1999) and *Leishmania major* which causes human leishmaniasis (Yang et al., 1990).

2.5 DNA vaccines

Expression plasmids

Viruses consist of protective membranes and protein shells in addition to the genome (RNA or DNA). Vaccination with whole viral particles hence always inoculates the individual with a number of proteins besides the immunogen(s) which are the basis for protective immunity. This is the case whether the virus is «live» or dead, attenuated, genetically modified or is a live vector. This augments chances of unwanted reactions in the vaccinated individual.

Very recently it was established that uptake and expression of naked, recombinant DNA molecules in the muscle cells of mammals and fish upon injection was a feasible option. It also became evident that expression of inserted genes from naked plasmids gave a very efficient immunological presentation of immunogens. With the optimal DNA-constructs, delivery systems and adjuvants, very efficient immunization against a number of disease agents was achieved in laboratory animals. Recombinant DNA molecules as well as the expressed immunogens were detectable in muscle cells for months. At the moment no such vaccines have been marketed, but the area is subject to intense research and clinical trials have been initiated for various vaccines in both humans and domestic animals (See Donnelly et al, 1997; Liu, 1998).

DNA vaccination

When the major breakthrough for naked DNA in a vaccination context was published (Ulmer *et al*., 1993), it created a great deal of media attention. Ulmer and his coworkers were working on immunisation against the nucleoprotein (NP) for the Influenza A virus. DNA which coded for NP was inserted in an expression plasmid which gives protein production intracellularly under the control of either an RSV (Rous sarcoma virus) or a CMV (cytomegalovirus) promotor/enhancer. A couple of weeks after injection of plasmid DNA directly into the thigh muscles of mice, antibodies against the nucleoprotein were found indicating that the gene had been expressed. The mice were then infected with a dose of influenza which is lethal for non-immune mice. However, 90% of the DNA-vaccinated mice proved to survive the infection.

That the mice were immune was one important observation. However, at least as impressive was the cross protection attained against different strains of the Influenza A virus. The ever new variants that arise are, of course, in a public health context, one of the major problems

associated with this virus. Antibodies developed following infection or vaccination with one variant confers no protection against another (Cohen, 1993). Ordinary influenza vaccines, which consist of chemically-killed virus particles, do not provide cross immunity, probably because it is first and foremost antibodies against surface structures in the virus that are developed. It is these structures which vary from one virus strain to another.

The same group of researchers reporting the original influenza studies subsequently refined the DNA vectors, thereby achieving still better genetic expression and even more reliable immunity (Montgomery *et al.*, 1993). Moreover, the same DNA vaccination strategy that was employed in the influenza studies has also achieved protective immunity against HIV 1 (Wang *et al.*, 1993), Hepatitis B (Davis *et al.*, 1993) and Rabies virus (Xiang *et al.*, 1994). A plasmid DNA which expressed the influenza virus hemagglutinin, was tested with a view to achieving protection against lethal viral infections in mice and chickens (Fynan *et al.*, 1993). This was partially achieved by intramuscular injections, intravenous inoculations and the use of aerosols containing a plasmid that were placed either in the nostrils or the trachea. However, by far the most effective immunisation was achieved using *biolistics*, small gold balls with DNA attached that were shot into the epidermis. This inoculation route could be used with tiny amounts of DNA compared with other routes. The same biolistic methodology, termed "genetic immunisation", has been employed to produce both polyclonal and monoclonal antibodies against several types of antigens (Williams *et al.*, 1991; Tang *et al.*, 1992; Barry *et al.*, 1994), and the method has subsequently been modified allowing simple, commercially available equipment to be used (Vahlsing *et al.*, 1994).

Generally speaking, vaccines based on purified proteins from viruses or bacteria, produced conventionally or by genetic engineering, as killed virus particles, first and foremost produce antibodies (humoral immunity) against the virus, but poor development of cytotoxic T lymphocytes which destroy virus-infected cells (cellular immunity) (Ulmer *et al.*, 1993; Wang *et al.*, 1993; Xiang *et al.*, 1994). The cellular immunity is broader than the humoral one, because the T lymphocytes overlook small differences between the virus strains, and for many viruses it has long been established that activated T lymphocytes stop or combat infection more effectively than antibodies.

Proteins which, with DNA vaccination, are expressed intracellularly from expression plasmids, are presented to the immune system in combination with tissue-type antigen class I molecules on the cell surface in exactly the same manner as takes place in a natural viral infection. DNA vaccination therefore gives the same type of immunity as that attained by undergoing a natural infection (Cohen, 1993).

Another advantage with DNA vaccination that has been cited, is that immunological defence is triggered against the protein(s) for which the plasmid codes, but seemingly not against the plasmid itself. Theoretically, this means that the same plasmid vector can be used repeatedly to deliver different genes, thus conferring protection to several different infectious agents (Cohen, 1993). This may envisage an "omnivax" vaccine which contains a cocktail of plasmids with genes from different pathogens, thereby providing protection against several infectious diseases.

When vaccination takes place with a simple plasmid vector, only the genes which one wishes to immunise against are expressed, whereas the use of viral vectors means that the individual will

be given several other biologically active genes (Barry *et al.*, 1994), and this is obviously, in principle, undesirable.

In contrast to recombinant proteins, which are produced in test tubes, proteins which are made by the animal itself following instructions given by the gene in the expression plasmid need not undergo extensive, expensive purification procedures (Cohen, 1993).

Relative to traditional vaccines which contain proteins, DNA vaccines are less dependent on cooling chains, because DNA is more temperature resistant than proteins. This is a very important practical aspect in those parts of the world where vaccination is really most necessary.

Vaccines have to be cheap, safe and effective. It is believed that in a short time a number of DNA vaccines will be developed which will satisfy all reasonable demands, and that they will become very widespread. During the very last years the DNA vaccination prospect has given high hopes for immunization against notorious killers as HIV (Letvin, 1998), various cancer forms (Benton and Kennedy, 1998) and malaria (Hoffmann et al., 1997).

DNA vaccines employ genes encoding proteins of pathogens or tumors, rather than using the proteins themselves, a live replicating vector, or an attenuated version of the pathogen itself. DNA vaccines consist of a bacterial plasmid with a strong viral promoter, the gene of interest, and a polyadenylation/transcriptional termination sequence. The plasmid is grown in bacteria (*E. coli*), purified, dissolved in a saline solution, and then simply injected into the host. The DNA is taken up by host cells, where the encoded antigen is made. The plasmid is made without an origin of replication that is functional in eukaryotic cells, prohibiting plasmid replication within the vaccinated host. The DNA vaccines are administered by direct intramuscular injection, although use of a "gene gun" to deliver gold beads onto which DNA has been precipitated is also under evaluation (Liu, 1998).

In a rapidly increasing number of animal models, DNA vaccines have been shown to be effective at generating protective immune responses against a wide variety of diseases (reviewed in Donnelly et al., 1997). The role of antigen presenting cells (APCs) in the immune responses that ensue upon expression of the encoded foreign protein has been studied for intramuscular (i.m.) immunization where the muscle cells are the primary protein-expressing cell type, but not for other routes of immunization such as that employing a "gene gun" (biolistics) to propel gold beads coated with DNA into the epidermis (reviewed in Donnelly et al., 1997). It has, however been demonstrated in many animal models that DNA vaccines can generate MHC class I-restricted CTL responses analogous to those obtained with live vaccine vectors. Furthermore, it is relatively simple to combine diverse immunogens into a single preparation, thus decreasing the number of vaccinations required, and in some instances the DNA itself seems to act as an adjuvant. Additionally, immunization with naked DNA vaccines results in protein synthesis in the definitive host. Such a protein is more likely to be similar or identical to the wild-type protein produced by a viral infection than many recombinant or chemically modified proteins. On the other hand, for bacterial antigens the mammalian post-translational modifications may result in antigens that differ from the bacterial versions, thus resulting in reduced immune responses (Liu, 1998).

DNA vaccination has the attractions of versatility, simplicity and potential economy, but there are various reservations (Lowrie, 1998). For example, in the present versions very small amounts of antigens are produced within the vaccinated individual. Furthermore, the vaccine DNA might integrate with nuclear DNA to generate tumors and other pathological conditions, and that is a real concern for skeptics, but not for believers (Donnelly et al, 1997 and 1998).

Vaccine vectors derived from positive-stranded RNA viruses

It has been demonstrated that the infectious cycle of positive-stranded RNA viruses can be launched directly from genome length cDNA copies precisely positioned within RNA polymerase II expression cassettes, when these are transfected into permissive cells (Driver et al., 1998). Vaccination of mice with plasmid DNA encoding infectious viral genome RNA from foot-and-mouth disease virus evoked antiviral immune responses (Ward et al., 1997).

A rapidly emerging area of vaccine research is the development of cDNAs derived from positive-stranded RNA viruses for the expression of heterologous antigens. A gene encoding the desired vaccine antigen has been substituted for viral structural or coat protein genes. Delivery of such constructs have been effected as *in vitro* transcribed RNA, plasmid DNA or various types of packed vector particles. The latter have shown promise for stimulation of broad antigen-specific immune responses in animal models (Pushko et al., 1997). There are now a number of publications in a variety of model systems that demonstrate the efficacy of nucleic acid- or particle-based vectors derived from alphaviruses (Pushko et al., 1997; Harihan et al., 1998; Berglund et al., 1998).

Oral DNA vaccines

As earlier discussed, it is essential to achieve mucosal immunity for many infectious diseases. At the moment there are intensive efforts being made to achieve DNA vaccines for oral use (Lowrie, 1998).

In one such approach the plasmid is propagated in a mutant strain of *Salmonella typhimurium* that cannot grow in vivo and the transfected salmonellae are taken as an oral vaccine (Darji et al., 1998). The live attenuated bacteria carry the vaccine DNA through the stomach, then through the M cells that cover the Peyer´s patches (PP) of the gut. From there the salmonellae enter APCs (macrophages and dendritic cells) where they die because of their mutation. This liberates multiple copies of the DNA vaccine right where they are needed, inside the APCs. In mice, this procedure led to extremely strong systemic immune responses to the DNA-encoded antigens expressed by the APCs. It was formally proven that the APCs, rather than the salmonellae, produced the vaccine antigens in this system (Darji et al., 1998). The expression appeared to continue long after the salmonellae had disappeared. Similar results have been obtained with mutants of shigella and invasive E. coli as carrier bacteria (Courvalin et al., 1995; Sizemore et al., 1997).

There are reports suggesting that diverse biodegradable carrier particles, e.g. poly-lactide-coglycolide microspheres, containing plasmid DNA can also make highly effective oral DNA vaccines (Jones et al., 1997). The trapped DNA seems protected from enzymatic degradation

and acid hydrolysis in the stomach and is carried through M cells in the PPs to be delivered to APCs by a route that exactly parallels that taken by salmonella.

The efficiency of the oral DNA vaccines indicates that there may be a normal process whereby large segments of intact DNA trapped in particles get from the gut, through PPs into the nuclei of APCs (Lowrie, 1998) and perhaps other cells in the organism (Schubbert et al., 1997; Doerfler et al., 1998).

2.6 RNA vaccines

RNA vaccines are not a new concept, although it appeared as that when the RNA vaccine against tick-borne encephalitis (TBE) virus was published in December 1998 (Mandl et al., 1998) and hit the headlines. Already in the first published article about a naked DNA vaccine it was noted that naked mRNA corresponding to the vaccine gene, elicited the highest immune responses when injected into mice (Wolff et al., 1990). During the following years other workers also reported on the use of naked RNA vaccines against selected cancer and viral antigens (Conry et al., 1996; Nair et al., 1998; Qiu et al., 1996; Zhou et al., 1994).

Development of the naked RNA TBE vaccine took advantage of an established fact for so-called positive strand RNA viruses, namely that the viral genomic RNA is infectious when introduced into permissive host cells. During the last years RNA from such viruses have been transcribed *in vitro* into cDNA clones, and this is also the case for the 11 kb long TBE virus RNA. Full length cDNA derived from TBE virus was *in vitro* transcribed into infectious RNA and this RNA was coated onto gold microcarrier particles and biolistically shot into the abdominal skin of mice by a GeneGun. RNA derived from a fully virulent TBE virus strain efficiently killed the mice, just as TBE virus itself. However, when infectious RNA from an attenuated TBE virus strain was used, the mice were fully protected against a later challenge with the virulent virus.

The authors of the report on TBE virus naked RNA vaccine (Mandl et al., 1998) interpret their results in a very optimistic way. The use of *in vitro* synthesized RNA corresponding to the genome of an attenuated virus combines the main advantages of conventional live virus vaccines with those of DNA immunization without some of the drawbacks of these methods. Chemically and biologically pure RNA may be produced without the need for cell cultures. The production does not require manipulation of infectious virus. Propagation of live, attenuated viruses, RNA viruses in particular, for live-virus vaccines can lead to phenotypic reversion to virulence. This requires extensive safety testing of each vaccine production lot, a danger which is claimed to be minimal when RNA derived from genetically stable, cloned cDNA is used. RNA are different from DNA vaccines in that there is no risk of chromosomal integration of foreign genetic material. When the infectious RNA has been internalized in host cells and viral replication initiated, all of the viral proteins are expressed and presented to the immune system of the host as they would be during a natural infection. This would include CTL responses, and hence RNA vaccines may have high efficacies and induce long-lasting, protective immunity.

2.7 Edible, plant «bioreactor» produced vaccines

In 1990, a group of philanthropic organizations led by WHO launched the Children´s Vaccine Initiative to set goals for developing vaccines that are safe, heat stable, orally administerd and widely accesible. These goals led to the idea of producing subunit vaccines in edible tissues of transgenic crop plants (Mason et al., 1992).

Ideally, vaccines against pathogens that cause respiratory, uro-genital or gastrointestinal diseases should sensitize the mucosal immune system (see section 1.2). There have been several strategies to induce mucosal immunity by delivering the antigens with live, replicating agents, such as attenuated or mutant strains of recombinant GM bacteria (e.g. *Salmonella typhi*) and viruses (e.g. adenovirus) (Mestecky et al., 1997). Most attempts have had limited success, and the use of live vaccines reaises safety issues. Nonreplicating subunit vaccines do of course offer alternatives, but they also have their disadvantages as outlined elsewhere in this report.

Transgenic, edible crop plants as production and delivery systems for subunit vaccines have a number of attractive advantages (Mason et al., 1992; Mor et al., 1998). Production would be as cheap as agriculture. Distribution would be as convenient as marketing fresh products. Vaccine administration would be as simple and safe as eating. To this end, crop plants have been genetically engineered to express viral or bacterial antigens in their edible tissues, and the antigens are then quantified and assayed for their immunogenic properties. An alternative approach is to infect susceptible plants with recombinant plant viruses, which harbor genes encoding antigenic proteins from infectious agents causing disease in humans or domestic animals (Mor et al., 1998).

Various plants have been genetically engineered to express subunits of infectious agents (Mor et al., 1998). A common experience, however, was that only relatively low levels of accumulated antigens, monitored as the percentage of total soluble proteins, were detected in the plants. The levels were ranging from 0.001% for rabies virus (RV) glycoprotein in tomato to 0.3% for cholera toxin B subunit (CT-B) in potato. Various strategies were therefore utilized in order to increase expression levels (reviewed by Mor et al., 1998). Strong plant promoters were selected to drive transcription of inserted transgenes. Plant 5´and 3´ untranslated regions that increase mRNA stability and translatability were employed. In some cases the coding regions themselves have been engineered to eliminate mRNA destabilizing sequences, undesired polyadenylation and cryptic intron sequences, as well as adapting DNA codon usage to that of higher plants. Optimization might also depend on targeting the protein to various possible subcellular compartments. But high expression has sometimes proven detrimental to the plants due to harmful effects of some of the antigenic proteins. Site-directed mutagenesis and inducible rather than constitutive promoters have been proposed as means to alleviate the toxicity of foreign proteins.

A number of experiments in mice have demonstrated that feeding with transgenic potato tubers expressing subunits of bacterial enterotoxins, i.e. LT-B, were partly protected against diarrhea when they were challenged with the intact toxins (Mason et al., 1998). Initially the expression levels were too low, implying that people would have to eat unreasonably large amounts of potato tuber to receive a desired dose of immunogen. But construction of a “plant-friendly”

synthetic LT-B gene improved expression levels considerably. These higher expression levels have allowed this “edible vaccine” to be tested in humans, representing the first human clinical trial of a plant-derived vaccine (Tacket et al., 1998). Volunteers who consumed 50 or 100 mg potato tubers developed specific anti-LT-B mucosal and systemic immune responses. The responses are comparable to those observed when humans are challenged with 10^9 enterotoxigenic E. coli bacteria (ETEC). At the moment studies proving protective immunity against ETEC following consumption of LT-B transgenic potato tubers have not been published.

Other mucosal immunogens are being evaluated for production in transgenic plants, including antigens from pathogens of the respiratory and urogenital tracts (Mor et al., 1998). Plants are one of the cheapest sources of protein, potentially also of recombinant proteins, which is particularly true for large-scale production. Most experiments so far have, however, used tobacco or potato for recombinant antigen production. But tobacco can not be a source for edible vaccines,while potato tubers, although tolerated by volunteers, are not palatable when uncooked. Bananas might be the ideal source of edible vaccines because they are consumed raw even by infants and are a major crop in many developing countries. Banana can be genetically transformed and modified, and the recent identification of pulp-specific promoters might allow specific expression of foreign proteins in transgenic bananas. Furthermore, seeds of plants such as maize and soybean have much higher protein contents than bananas and tomatoes and express very high levels of foreign proteins. They can be preserved for long periods and are often consumed raw, either whole or ground into flour, thus suggesting that hey might be a good source for some edible vaccines.

3 What is “risk”?

The term “risk” is very often confused with “probability”, and hence used erroneously. Risk is defined as the probability that a certain event will take place multiplied by the consequences arising *if* it takes place. The atomic bomb makes a good basis for conceiving the contents of the term. With the regard to development and commercialization of genetically engineered nucleic acids, organisms and viruses we often are neither able to define probability of unintended events nor the consequences of them. Hence, the present state of ignorance makes scientifically based risk assessments impossible. This calls for invoking the “Precautionary principle”.

4 The “precautionary principle”

This principle is now established in international declarations and agreements. It was introduced as an ethical road sign. The principle implies that responsibility for future generations and the environment is to be combined with the anthropocentric needs of the present.

In the context of gene technology and use of GMOs a general definition might be:
“ In order to obtain sustainable development, politics should be based on the precautionary principle. Environmental and health policies must be aimed at predicting, preventing and attack the causes of environmental or health hazards. When there is reason to suspect threats of serious, irreversible damage, lack of scientific evidence should not be used as a basis for postponement of preventive measures” (revised after Cameron and Abouchar, 1991). A comprehensive discussion of the Precautionary principle in the context of genetic engineering is soon available (Myhr and Traavik, 1999).

The value of the Precautionary principle both for risk management and for generation of risk-associated research, can hardly be overestimated.

In the last decade, researchers have been eager to *make plants resistant to viral infections* by inserting virus genes in the plant genome. If, for example, the gene which codes for the coat protein for the cowpea chlorotic mottle virus (CCMV) is inserted in plants, the plants become resistant to both CCMV and several other related viruses. It has now been shown that when such transgenic plants are infected with other viruses new, recombinant viruses can arise which have had their host specificity and other biological properties changed (Greene & Allison, 1994). This possibility, and the necessity of investigating it, had been pointed out by critically inclined scientists for many years, but their protests had been drowned by representatives of both the biotechnological industry and the research community optimistically eager to develop the technique. Even after the publication of Greene and Allison’s results, such experts attempted to undermine the significance of the discoveries without having alternative results of their own to point to (Falk & Bruening, 1994). Incidentally, the history did not end here. In further work Greene and Allison demonstrated that a targeted trimming of the viral transgene seemed to eliminate development of viral recombinants (Greene and Allison, 1996). This illustrates the importance of invoking the “Precautinary principle”, to gain time for identification of risk imposing mechanisms and look for means to prevent them.

5 Potential environmental risks and hazards related to genetically engineered vaccines

It must be emphasized that the risk factors and hazards to be discussed are hypothetical, based on theoretical considerations. There is, however, a valid and definite reason for this. A vast number of research articles concerning vaccine safety as related to adverse immunological and other reactions in vaccinated individuals have been published. But up to December 1998 scientific literature concerning environmental and ecological risks is very sparse, and exclusively related to the use of live virus vector and deletion mutant vaccines (for review, see Sandvik and Tryland, 1996).

5.1 Synthetic and recombinant vaccines

These products simply contain pure proteins (immunogens, antigens). The only thinkable risks associated with unintended releases of such vaccines will be toxic, allergic and other unwanted immunological reactions in animal or human individuals within the release area. However, if the DNA constructs that are used to produce recombinant vaccines, or the cell cultures used to have them expressed, are released or escape from laboratories/manufacturing units, they will represent the same potential hazards as any other genetically modified nucleic acids or organisms (see below).

5.2 Live virus vaccines

Viruses - biology, ecology and risk
Combat of invertebrate invations and vaccination of wildlife mammals are executed by release of GMVs, while immunization of domesticated animals or humans with live GMVs pose varying chances of escape.

Introduction of new viruses into any ecosystem carries risks, which are related to the general characteristics of viruses. The risks may be augmented or diminished by the specific characteristics of the virus to be introduced.

Implantation of geneexpressing and/or replicating GMVs into an ecosystem poses special theoretical hazards, some of which may be impossible to predict.

Viruses multiply intracellularly in permissive host cells. One single virus particle infecting a permissive cell may give rise to millions of new particles during a short time (hours to days). In addition to such fully productive infections, some virus/host cell combinations may result in

persistent infection with virus shedding for extended periods, while others lead to latent infection with viral DNA in a host chromosome-integrated or episomal state. Latent infections may be intermittently reactivated and accompanied by virus shedding. Integration of viral *DNA* into the host cell genome may by itself have harmful consequences, irrespective of viral gene expression or replication.

The host tropism, at the species-, organ- or cell type-level, is quite narrow for some viruses, while others have a much wider host-spectrum. For most viruses the molecular mechanisms determining host-cell specificity are not known in detail. Restrictions may be present at various steps during a virus multiplication cycle, from the lack of cell membrane receptors to subtle incompatibilities with host cell enzymes necessary for viral nucleic acid transcription and replication.

For many virus/host cell combinations permissivity is a relative term, since it may be influenced to a considerable extent by the menu of genes expressed by the cell, and by the levels of gene expression. In culture, the permissivity of a given host cell may be manipulated experimentally by activation of intracellular signal transmission pathways, i.e. by hormones, growth factors, cytokines etc. Such procedures may also enhance persistent or reactivate latent infections. At the intra- as well as at the inter-species level of host animals this is illustrated by a vast variation in susceptibility for a given virus strain. Such variation may be related to sex, age, mating season, pregnancy, genetic differences, infection with other viruses or micro-organisms, and environmental factors promoted by season or by pollution

It is important to be aware the distinction between viral infection and viral disease. An infected individual may shed virus and represent a transmission reservoir without showing clinical symptoms. Yet, other individuals within the same or other species may become clinically ill, or the viral infection may result in abortions, stillbirths, teratogenic or oncogenic effects. For persistent/latent infections, clinical symptoms may be present intermittently, only under special circumstances, or appear a long time after infection.

Different strains of the same viral species may have different pathogenicity, as well as host-cell or -species tropism. Even genetic differences at the single point mutation level may result in virus strains with aberrant phenotypic characteristics.

For GMVs it is hence conceivable that unintended phenotypic characteristics with unwanted ecological consequences are established in addition to the intended modification(s). This may not become evident unless very comprehensive and carefully planned experiments are carried out. In many instances fully adequate experiments are totally precluded by the complexity and the regular or occasional variations of the recipient ecosystem.

Ideally, before any GMV becomes implanted into a new location/ecosystem a number of crucial questions should be answered (see 5.2.2. and 5.2.3.). Some of these questions deal with the biological and phenotypical characteristics of a supposed genetically stable GMV. But the situation becomes even more complex and unpredictable if the *GMV* parental strain under certain conditions or circumstances is genetically unstable, giving rise to viral strains with altered characteristics.

Genetic recombination between an implanted GMV and naturally occurring relatives already circulating within the recipient ecosystem, may be an important mechanism for the emergence of geno- and pheno-typically altered virus strains.

5.2.1. Unintended spread

In some instances it has been demonstrated that minor genetic changes in, or differences between, viruses can result in dramatic changes in host spectrum, permissivity and pathogenic potential. For most viral species the nature, location and interplay of genes determining host specificities and pathogenic potentials are virtually unknown. Hence we have no chance to predict how our gene modification, i.e. point mutation(s), deletions, insertions, may affect the transmission of a virus within and between different species (Mulder, 1997). The only way we can minimize such potential risk factors, is by performing well-planned "microcosms" experiments which include ecosystem imitation, host organisms, vaccine viruses and naturally occurring relatives. If the GMV is breaking assumed species barriers, replication in new hosts may lead to emergence and selection of secondary, spontaneous genetic changes and mutations which is their turn may influence transmission abilities, host preferences and virulence. Such changes may take place and accumulate over time, and may not be detected during short-time experiments.

Most adenoviruses seem to have rather restricted host-ranges, although this has not been systematically examined in most cases. To circumvent this host-species restriction, investigators reported the construction of recombinant adenoviruses containing host-range mutations allowing human adenoviruses to infect non-permissive host cells (Cheng et al., 1992; Caravakyri et al., 1993). But this obviously make the resultant vectors more risk-prone in an ecological and environmental context. It should also be borne in mind that adenovirus excretion from the pharynx could be demonstrated following enteric inoculation in human volunteers (Schwartz et al., 1974). Environmentally resistant viruses, like adenoviruses, may be spread over amazingly large areas in aerosols created from the repiratory organs of virus-excreting individuals.

5.2.2. Non-target effects

Even when a given GMV vaccine are advantageous in every thinkable way in the intended vaccine species, it may be detrimental on a total basis due to its effects on unintended non-target species, and hence on the ecosystems such species are parts of. This may relate to acute disease symptoms, but also to persistent infections, which interfere with reproduction and behaviors. Genetic differences between strains and geographical variants of the same animal species may influence the relative effects of the GMV infection. Over time, new or spontaneous genetic changes in the GMV may modulate the interplay with host species in new, unpredictable ways.

5.2.3. Genetic "pollution"?

It is possible that a given GMV may cause "pollution" of genomes in related, naturally occurring viruses, or in the DNA of host cells. Our insight into naturally occurring relatives of viruses we are already vaccinating against, or are going to vaccinate against in the future, is very limited. Theoretically, different GMVs may by different mechanisms (i.e. recombination, reassortment) exchange nucleic acids and genetic information with related viruses. This may

result in hybrid, recombinant viruses with unpredictable characteristics. If a GMV or a hybrid is able to integrate its DNA into host cell chromosomes in some species, this may have dramatic biological, and hence ecological, effects in a short or longer time-span. Gene expression patterns and functions of host cells may become influenced by the integrated DNA, but also by viral or chimeric proteins translated from integrated DNA.

When GMV particles are broken down in the environment, naked nucleic acids will be released. In such situations horizontal gene transfer of GMV genomes, or parts thereof, is a potential hazard by the same token as any other genetically engineered nucleic acid (reviews: Nielsen et al., 1998; Traavik, 1999).

5.2.4. Genetically modified viruses for homologous immunization

Although gene-deleted viruses were initially considered as a promising strategy, recent experience with an AIDS-related vaccine have raised serious concerns about both target and non-target effects of such vaccines. A vaccine made by deleting several genes from the Simian immunodeficiency virus (SIV), supposed to be safe, caused AIDS in infant and adult macaques (Baba et al., 1999).

To illustrate some of the theoretical hazards, and the lack of key knowledge for risk assessments, I have chosen to use gene-deleted Pseudorabies virus vaccine as an example.

Example: *Gene-deleted Pseudorabies vaccine*

The alterations
The vaccine virus was developed by genetic engineering of a live, attenuated PRV strain. The vaccine virus is a multi-deletion mutant. The main deletion (2055 basepairs) is situated in the small unique (Us) part of the genome. It removes gE and part of the so-called 11K protein-gene. A 2 basepair deletion has been introduced into the *tk* (thymidine kinase) gene, which is hence left non-functional, i.e. the vaccine PRV has a TK÷ phenotype. Finally, a deletion has removed 73 basepairs from a non-coding control region in the repeated sequences. The gE-deletion was selected for after a targeted, homologous recombination in cell culture, while the small *tk* gene deletion was achieved by genetic engineering.

The effects.
gE seems to be an essential protein in transneuronal spread of PRV. Studies have shown that gE-negative PRV replicates in peripheral tissues, infects first-order neurons and spreads towards the CNS via both the olfactory and trigeminal routes (review by Mulder et al., 1997). The second- and third-order neurons in the porcine CNS do, however, seem to be less efficiently infected by a gE-negative than a wild-type PRV (Jacobs, 1994; Kritas et al., 1995). But there may be considerable differences in the effect of gE-deletions between different neuron circuits in the pig, and also between pigs and other permissive animal species (Cord et al., 1992; ter Horst et al., 1993; Whealy et al., 1993; Standish et al., 1994).

The PRV-encoded thymidine kinase (TK) is not considered essential for growth in dividing cells, but seem to be required for productive infection of non-dividing cells such as neurons and resting peripheral blood mononuclear cells (Mulder et al., 1995 and 1997).

Pseudorabies: the disease
Pseudorabies virus (**PRV**; synonyms *Aujeszky´s disease virus* and *herpesvirus suis type 1)* causes neurological disorders in pigs, which is a natural host, as well as in a number of other domestic and wildlife animal species. The disease in pigs results in vast economic losses as well as other practical problems, i.e. having trade barriers created between countries. Control of the disease and eradication of PRV, with or without the use of vaccines, consequently have high priorities worldwide.

Pseudorabies: prevention and control
The epizootiology of PRV is complicated. The prospects of healthy virus carries and -shedders, latent infections that may be reactivated with or without accompanying clinical symptoms, vertical transmission from sow to offspring etc. must be taken into consideration. Campaigns to prevent disease, and ultimately to eradicate PRV from swine populations have been mounted with or without the aid of vaccination (Stegeman et al., 1994). Until recently the UK eradication programme was based on serological surveillance, culling of seropositive animals and the control of pig movement (Minson, 1989).

Several types of PRV vaccines are available (Kimman et al., 1995; Mulder et al, 1997):

- Killed whole virus vaccines
- Subunit vaccines
- Conventionally attenuated live vaccines
- Genetically engineered live vaccines

In general, live vaccines are more potent than the dead ones, especially in mounting an efficient cellular immune response. Conventionally attenuated live vaccine PRV strains have, however, been shown to contain mutations that may reduce immunogenicity. There is also the risk that such PRV strains may revert to full virulence. These were the main, expressed reasons why several research groups started to develop genetically engineered vaccine viruses with defined deletions in the genes encoding glycoproteins E (gE, formerly gI), C (gC) or G (gG) (Kit et al., 1987; Marchioli et al., 1987; Moorman et al., 1990). In order to enhance their safety, TK which enhances viral DNA replication has been deleted from these engineered PRV strains (Mulder et al., 1997).

In eradication campaigns, gE-deleted vaccine strains have been used in conjunction with a serological test that specifically detects antibodies against gE. This makes it possible to identify vaccinated individuals that have become infected with wild-type PRV (van Oirschot et al., 1990; Kit, 1990).

<u>**Concerns connected to genetically engineered PRV vaccines**</u>

PRV transmission
So far short-term economical interests have governed vaccine development, i.e. to avoid losses in slaughter weight have been more important than eradication of PRV. The vaccines that have been introduced so far, including the gE-/*tk*-deleted vaccine, are not able to stop transmission of wildtype PRV strains, and the vaccine strain itself may spread to contact pigs, setting the stage for single- or multi-step recombinational events (Bouma et al., 1997b; Parker et al., 1997; Bouma et al., 1997a; de Smet et al., 1992; Mulder et al., 1995). Gene-deleted pseudorabies vaccines have now been shown able to infect sheep (see below).

Non-target effects

It is well known that a number of domestic animals and wildlife species are susceptible to PRV infection and may contract dramatic and even fatal disease (Kimman et al., 1991; Kit, 1994). It is also well established that there are striking differences in virulence for the same wildtype PRV in different host species, and between different PRV strains in the same host species. The molecular basis for these aberrations is unknown (Christensen and Lomniczi, 1993; Bouma et al., 1996). There are indications of human infections during out-breaks in pigs or cattle (Anusz et al., 1992). Wild boars are naturally PRV-infected reservoir animals and are experimentally susceptible for porcine PRV (Oslage et al., 1994). For other wildlife animal species no systematic studies of PRV susceptibility have been published. Neither are there any published reports with regard to natural occurrence of PRV or PRV-like viruses in wildlife species. Whether PRV deletion-mutants are able to infect wild life species is also unknown. Such knowledge is essential for risk assessment concerning recombination between PRV vaccines and naturally occurring viruses.

In a recently reported case (Jacobs et al., 1997), sheep housed together with pigs started to die from PRV infection. Pigs were vaccinated by the 783 gE-/*tk*-vaccine and sheep with the live attenuated Bartha vaccine. In spite of that, sheep continued to die. Both vaccine viruses as well as wild-type PRV were detected in the sheep, the latter in extremely small amounts. The authors suggested that it was wt virus that killed, but this was by no means proven. Neither were data on recombinations between the 3 involved viruses offered, which I find most unfortunate.

In 1992 gE-deleted PRV vaccines were named the presently preferred choice by an expert group (Pensaert et al., 1992). Serious doubts were, however, raised about the efficacy, and the experts concentrated on target-effects. No demands for documentation concerning non-target effects were expressed. This situation was still valid in 1997 (Mulder et al., 1997). The European Pharmacopoeia has formulated safety requirements for live PRV vaccines (Kimman, 1992). These requirements do not include a number of those hazards that are most important from environmental and ecological points of view.

- Reduced virus excretion after experimental reinfection of vaccinated animals.
- Efficient immunization and lack of latent infections.
- Significantly reduced PRV transmission under natural conditions.
- Susceptibility of non-target species within surrounding ecosystems.
- Potential for recombinations between vaccine virus and naturally occurring PRVs and PRV-relatives.

These requirements are especially important for Norway, since this is one of the few countries of the world where PRV is not enzootic at the moment (Kit, 1994).

Latent infections

The definition of such infections is that the virus is present intracellularly somewhere in the host, but the virus genomes are dormant, i.e. not transcribed or replicated. Latency may be broken, for the viral genomes to become active, when the host cells receive new signals from endogenous or exogenous sources, for instance in connection with physical or psychological stress, disease or endogenous hormone fluctuations.

PRV, as many other herpesviruses, are frequently establishing latency after primary as well as secondary infections (Ben-Porat et al., 1985; Tham et al., 1994; Mulder et al., 1997). To definitely reveal or exclude latent PRV infections it is necessary to employ sensitive PCRs (polymerase chain reactions). Efforts to isolate virus in cell culture are not good enough. Virus isolation failed for 11 PRV-convalescent pigs. By PCR latent infections were detected in all animals (Wheeler and Osario, 1991).

There are various important questions concerning latency and gene-deleted PRV that have not at the moment been answered in a satisfactory way.

- Recent studies indicate that gE-deletion results in a diminished invation potential for some, but not all, types of neurons (Enquist et al., 1994; Mulder et al., 1997).
- Deletion-mutants may in some instances act as defective interfering (DI) viruses which may contribute to establishing persistence of wildtype virus during co-infections (Roux et al., 1991), or result in unexpected angmentation of cytopathogenicity and acute disease (Tautz et al., 1994).
- The deletion of 73 bp from a promoter/enhancer sequence in the Us region of the gene-deleted PRV vaccine, may mean that important transcription factor binding sites have been removed. This may result in unpredictable changes, increase or decrease, in viral gene expression and multiplication in different host cells and species. Such possible biological effects can only be revealed by experiments. The prospects and risks carried by latency/reactivation events have not been satisfactory clarified for the gE-/*tk*-deleted PRV vaccine.

Recombination
This expression implies that 2 PRV strains infecting the same host cells may exchange parts of their DNA genomes, so that DNA pieces from both parents are joined together (recombined) to make new, hybid off-springs with unforecastable biological properties (Henderson et al., 1991; Dangler et al., 1993; Glazenburg et al., 1995). Little is known about properties that determine cell or host species tropism.

Several experimental studies have demonstrated that coinoculated modified live PRV vaccine strains could recombine in vivo to create virulent recombinant strains (Henderson et al., 1990, 1991; Katz et al., 1990; Dangler et al., 1993; Glazenburg et al., 1995; review by Mulder et al., 1997).

The potential risks represented by recombination events have not been satisfactory clarified for the gE-/tk- deleted PRV-vaccine.

Conclusion
The uncertainties and unpredictability linked to transmission, non-target effects, latent infections and recombination events for gene-deleted PRV vaccines are quite considerable.

5.2.5. Live virus-vector vaccines

Example: Recombinant, live VV/rabies vaccine

Risk assessment and monitoring in relation to VV/rabies vaccine release

Relatively thorough risk assessment and monitoring were performed before, during and after release. The investigators concluded that the effects on the size of the rabies virus-reservoir and the distribution and dissemination of virus were positive (Brochier et al., 1991; Pastoret and Brochier, 1996; Brochier et al., 1995).

The investigations have, however, been met with harsh criticism from different quarters. The most serious criticism has been that the investigations have centered around target effects of vaccination, and that the studies of ecological non-target effects have been too limited both with respect to extent and penetration (Kaplan, 1989; McNally, 1994).

Target-effects, in this connection, mean the goals one intend to achieve, i.e. immunization and fight against rabies. Even these results have been met with skepticism. It has, for instance, been pointed out that occurrence of rabies are naturally cycling among foxes, and that outbreaks consequently will vary with the population densities. Seemingly, this has not been taken into account during the investigation design (Anderson, 1991; McNally, 1994).

General risk factors connected with release of live recombinant VV/rabies vaccine

Most potential hazards may be categorized under the heading «non-target effects». These may be due to the released virus itself. The innocuousness of VV/rabies recombinants is by no means proven (Hanlon et al., 1997; Zhen et al., 1996; Moos, 1995). But equally possible is the emergence of new virus strains as a result of recombination events between vaccine virus and a naturally occurring relative taking place in a double-infected individual. Such hybrid viruses may have totally unpredictable characteristics with regard to host species susceptibility and virulence in different animal species.

If a genetically modified orthopoxvirus infects an individual, animal or human, which already carries another orthopoxvirus, a hybrid progeny virus with unpredictable pathogenicity and altered host range might be the outcome. Such worst case scenarios include new emerging diseases and ecological catastrophes. In our lab we have shown that some orthopoxviruses circulate among small rodents in Norway. In addition, Norwegian orthopoxvirus strains, isolated from a clinically ill house-cat and a woman, show genetic characteristics in common with both vaccinia virus and cowpox virus. It is important to gain knowledge about the biological and genetic diversity among the circulating Norwegian orthopoxviruses, as well as about their ecology and reservoir species. It is also important to verify whether the two Norwegian isolates represent hybrid viruses due to recombination events. Finally, one needs to investigate the recombination potential between naturally occurring- and genetically modified orthopoxvirus in authentic Norwegian host animals.

For VV there is little relevant knowledge concerning these mechanisms (Moss, 1996; Fenner, 1996). Some experts have voiced the opinion that there is a number of questions that have to be answered in a satisfactory way before any virus should be released:

- Can the virus engage in genetic recombination, or by other means achieve new genetic material? If so, will the hybrid offspring have changed their host preferences and virulence characteristics?
- Can other viruses that are present within the ecosystem influence the infection with the released virus or its offspring?

- Can insects or migrating birds or animals function as vectors for the released virus or its offspring, to disseminate viruses out of their intended release areas?
- For how long can the virus and its offspring survive outside host organisms under realistic environmental and climatic conditions?
- Is the virus and its offspring genetically stable over time?
- Can the virus or its offspring establish long-lasting, clinically mute, persistent or latent infections in naturally accessible host organisms?
- Can the virus or its offspring activate or aggravate naturally occurring latent or persistent virus-infections?

Most of these questions are unaccounted for as related to genetically engineered VV. Even when they have been answered by experimental investigations, ecological non-target effects can not be excluded because even carefully designed model studies will not directly reflect the real ecosystem conditions, which in addition are dependent on local variable parameters. However, some warning signs have already been seen:

- During the human small pox eradication campaign, VV found a new host species and established itself in a new reservoir, namely the buffalo (Dumbell and Richardson, 1993).
- It is a general experience that inserts may change the virulence and host preferences of viruses (Mulder et al., 1997).
- MRV (Malignant rabbit virus) seems to be a recombinant between SFV (Shope fibroma virus) and myxoma virus. It seems to have arisen by mixed infection in wild rabbits. MRV causes an invasive malignant disease and profound immunosuppression in adult rabbits, much more serious that disease caused by any of the parental viruses (Strayer et al., 1983). MRV has received more than 90% of its DNA from one parent (myxoma virus) in a coupled recombination and transposition event (Block et al., 1985). The MRV story exemplifies the unpredictability of virus recombinants with regard to biological characteristics and virulence.
- A recombinant field isolate of capripoxvirus has also been detected (Gershon et al., 1989). The new virus was the result of recombination between a capripoxvirus vaccine strain and a naturally occurring virus strain.

Comments to risk assessment investigations performed

Compared to the prospects of long-term ecological effects, there are serious inadequacies in the investigations done:

- It has been attempted to detect virus-dissemination, but only by isolation in cell cultures, which demands large amounts of virus, not by PCR.
- Virus investigations with regard to the bait-eating, non-target species which were identified (rodents, birds, insects) have not been performed properly (Boulanger et al., 1996; Hanlon et al., 1993).
- Attempts to detect and characterize naturally occurring relatives of VV in the areas of bait-release have not been properly carried out (Boulanger et al., 1996; Pastoret and Brochier, 1996; Boulanger et al., 1995). For that reason advance assessments of recombination risks could not be executed (see below about the situation in Norway).
- All investigations carried out concern short-time effects, i.e. short-term studies for long-term effects!
- The genetic stability of the VV/rabies recombinant vaccine virus has been rather superficially tested in monkey kidney cells and a laboratory mouse strain. It should of

course have been tested in authentic host animals. In addition, stability was not tested by sensitive PCR-sequencing methods.

Naturally occurring poxvirus relatives of VV in Norway

Very recent PCR- and serology-based investigations in Norway (Sandvik and Tryland, 1996; Sandvik et al., 1998; Tryland et al. 1998a,b,c,d,e; Hansen et al., 1999) have demonstrated that orthopoxvirus(es) closely related to VV are widely distributed with regard to geography, ecosystems and host animal species. Approximately 20% of shrews and small rodents belonging to 8 species are carrying orthopoxvirus(es) in one or more of their organs (lungs, kidneys, liver, spleen) at a given time-point, and a similar proportion have specific antibodies as a sign of past infections. The DNA sequences of two different genes (tk and atip) demonstrate that the Norwegian viruses are so closely related to VV that recombinations and hybrid offspring in doubly infected animals is a very real prospect. Experiments to clarify this prospect are now (1999) being performed by mixed infections in an authentic orthopoxvirus host animal (bank vole, *Clethrionomys glareolus*) and Balb/c mice.

Avipoxvirus vectors

The established dogma has emerged that avipoxviruses do not replicate, and are hence safe for use, in mammalian hosts (Fries et al., 1996; Baxby and Paoletti, 1992; Taylor and Paoletti, 1988; Taylor et al., 1988). This is an extreme example of narrow singletarget-based safety assessment. Going back to the original papers from which the cited dogma originates, one does not find any definite foundation for it. A very restricted number of avipoxviruses (fowlpox, canarypox and pigeon pox) have been involved in infections of a very restricted number of mammalian species and cell lines of mammalian origin. Non-target wildlife mammals have not at all been investigated. Neither has the prospect of recombinations between genetically engineered avipox vectors and naturally occurring avipoxvirus species been touched. The genetic diversity of avipoxviruses in different bird species is not well investigated, and may be much greater than realized at the moment.

In my lab, we have made a total of 11 poxvirus-isolates from Norwegian birds. PCR-amplification of the *tk* gene, which is considered very highly conserved among avipoxviruses, in comparison with two different reference avipoxvirus-strains (ATCC VR229 and VR251) demonstrated the identity of only 3/11 Norwegian strains. Circumstantial evidence indicated that these disappointing results were due to variation in the *tk* gene sequences of the various virus isolates, and that the genetic diversity among avipoxviruses may be greater than appreciated so far. These experiments hence illustrate the urgent need for penetrating studies designed to elucidate the biology, ecology and genetics of avipoxviruses before they are being used as vaccine vectors under uncontained conditions.

5.3 Bacterial vectors

Much basic work is needed before the recombinant bacterial vectors may be taken into practical use. From some points of view they have produced conflicting an d unpredictable results so far. In addition, since most of these vectors are designed for mucosal administration, the issue of oral tolerance needs to be addressed. The length of residence of a given recombinant organism within the host is also a matter of concern (Jones, 1998). Most attenuated pathogens remain in the host for a few days to weeks. It is, however, not known whether introduction of foreign

genes may change the relationships between vector and host. Another worrying aspect is the unpredictability of gene expression level. During studies of genetically engineered *Salmonella/Leishmania major* vectors in mice it was revealed that a *low* antigen dose regimen was necessary to establish stable, protective CTL immune responses in susceptible mice, whereas *high* doses elicited only humoral responses and exacerbated the disease (Bretscher at al., 1992).

It has recently been demonstrated that GM bacteria may transfer their transgene efficiently to indigenous bacteria in the mammalian gut (MacKenzie, 1999). This possibility has not been investigated for the bacteria which are now being genetically engineered as oral vaccines.

If genetically engineered bacterial vaccine vectors are released or escape to the environment, their DNA may be spread by the same processes as any other DNA (reviews: Nielsen et al., 1998; Traavik, 1999).

5.4 DNA vaccines

DNA vaccines are composed of shuttle DNA vectors. They are constructed to replicate, and sometimes also express, their genes within a vast number of eukaryotic as well as prokaryotic cell types. Upon release or escape to the wrong place at the wrong time, horizontal gene transfer with unpredictable long- and short-term biological and ecological effects is a real hazard with such DNA constructs.

There is now growing concern over some aspects of using naked DNA vectors within the fields of vaccinology and gene therapy, and there is a growing debate over the potential for generating infectious viruses and harmful effects due to random insertion into the cellular genome (Brower, 1998; Jane et al., 1998; Putnam, 1998). Recombinant DNA vaccines, in both the naked and viral form, tend to be unstable and prone to recombination, increasing the likelihood of generating new viruses (Ho et al., 1998).

Gene expression from naked DNA in multicellular organisms

Like many other significant discoveries, this, too, happened by chance. What had prevented an earlier breakthrough was a dogma that naked DNA introduced to an intact animal organism would very quickly be broken down and would lack biological importance. The scientists who had this dogma removed (Wolff *et al.*, 1990) were really aiming to test chemicals which could raise the uptake of naked DNA in the muscle cells of living mice. It concerned simple shuttle vectors used in laboratories all over the world.. One of the obvious verifications in the trial protocol was that chemicals should be excluded, naked DNA alone being injected intramuscularly. It then transpired that the muscle cells of these creatures efficiently took up DNA and produced larger quantities of the protein for which the plasmid coded than was found in the mice that were treated with chemicals. Naked RNA was also injected during the same trials, and those mice produced the absolute largest quantities of protein (Wolff *et al.*, 1990), but that discovery, surprisingly enough, was not immediately followed up.

In the wake of this pioneering work, but also independent of it, a number of important studies were carried out which showed that genetic expression could be achieved following the injection of mice with simple expression plasmids containing genes with potential therapeutic

application (reviewed by Donnely et al., 1997). The possibilities for directed genetic expression using such strategies was also demonstrated in several species of animals. For instance, it was shown that if young, growing up *carp* (ca. 10 g) received an intramuscular injection of plasmids containing reporter genes (_-galactosidase, CAT) under the control of various promoters (including human, viral and rabbit), a powerful genetic expression was obtained (Hansen *et al.*, 1991). Intravenous injection of naked, circular virus DNA into rabbits and mice gave a powerful genetic expression with the development of antibodies and the production of new virus particles (Fredriksen, 1993; Fredriksen *et al.*, 1994).

It proved in practice, for so far unexplained reasons, that there seemed to be major differences between the plasmids used with regard to breakdown in the organism and/or uptake into various cells and organs. Only injection into musculature proved to give a reproducible genetic expression from naked DNA. However, it was gradually demonstrated that if mice were given intravenous injection of plasmids in liposomes, this was able to give expression in several organ systems (Zhu *et al.*, 1993) including the ovaries, certainly a highly undesirable result because a possible integration of plasmid DNA in the chromosomes of the sex cells can lead to the inheritance of a genetic change. The same problems have now been highlighted in further gene therapy trials in animals as well as humans (Boyce, 1998). Uptake of naked DNA by sperm cells of marine organisms and mammals have been established, and transgenic animals created. It is indeed contemplated using sperms to deliver therapeutic genes (Spadafora, 1998).

Subsequently, using intravenous injection, or local installation in the respiratory passages, scientists achieved *in vivo* gene transfer to rabbit lungs of a plasmid which contained the gene for recombinant human alpha l-antitrypsin, driven by a CMV (cytomegalovirus) promoter, in complex with cationic liposomes. Both insertion routes gave expression in the lungs for at any rate 7 days (Canonico *et al.*, 1994).

A careful study should be made to determine whether genetic expression from liposome-plasmid complexes following installation in the respiratory passages is a common phenomenon which can happen to plasmids that go astray. It is undesirable because such expression may lead to serious reactions in the respiratory passages.

It has not been conclusively determined whether the various plasmid vectors that may be used can become integrated in the chromosomes of the planned or accidental target cells. The integration tendency will probably vary for the same plasmid in different target cells and for different plasmids in the same type of target cell.

If naked DNA vaccines prove as advantageous as preliminary results imply, the use of naked DNA and the proportion of it which ends up in the wrong places will necessarily increase greatly in the years to come.

Persistence of DNA in the environment

This is a key problem in connection with pre-assessments of damaging effects and includes not only the power of resistance of nucleic acids generally, but also the length of fragments which can persist for how long under what conditions. Even though this may seem very simple put like this, it is a very complex field of research. Specific properties in the nucleic acids concerned clearly play a major role, and in addition the field covers mutual impact between a number of

freely varying environmental factors (for recent review, see Nielsen et al., 1998; Traavik, 1999).

The new branches of science, molecular palaeontology and molecular archaeology, show quite clearly that relatively long chains of chromosomal DNA can survive for a long time under certain conditions. Even without considering Jurassic Park, 65 million years, etc., there is proof of survival over thousands of years (Pääbo *et al*., 1988). Controlled biochemical studies concerning the breakdown of DNA in solution under "normal" conditions imply that DNA generally will be severely degraded, if not totally broken down, after 40-50,000 years (reference in Morell, 1993). However, it is, as we know, one of the inherent curses of science that Nature only seldom views it as a priority to reproduce or mimic "normal" laboratory conditions!

When it comes to survival of DNA under natural environmental conditions, on the whole little research has been done in this important field. Moreover, most of the reported trials have used pure, homogeneous clay and sand as the DNA recipient, and these have completely different properties from the far more heterogeneous and complex, naturally occurring soils. This is illustrated by a published study (Ogram et al., 1994) which shows the dramatically differing extents in which varying lengths of DNA fragments (from about 2 to 23 kbp) can be adsorbed by various types of soil. This work also very convincingly demonstrates how adsorption to solid surfaces can have major consequences for DNA survival, because different types of soil particles give varying degrees of protection from DNase attack. Even the largest fragments, under favourable circumstances, could be recovered intact after several weeks. Romanowski *et al*. (1993) also showed that the type of soil is important and demonstrated the continued existence of transformable plasmids 60 days after release. Recorbet *et al.* (1993) demonstrated the persistence of substantial quantities of chromosomal DNA from genetically modified *E. coli* 60 days after the bacterial culture had been inoculated in natural soil. This was much longer than bacterial cells could be detected by plate counting and immunofluorescence. Widmer et al (1997) found persistent transgenic plant from tobacco and potato) marker gene *nptII* in soil for 77-137 days.

Free DNA has been found in all the ecosystems (sea water, fresh water, sediments) so far investigated (Lorenz & Wackernagel, 1994), even though DNases are widely distributed. Pooled data acquired by various methods show that such DNA is present in significant amounts, most of it having a microbial origin. It has been demonstrated that various bacteria liberate naked plasmids and chromosomal DNA to the surroundings during spore formation, during competence development in cells that are very much alive and when cells are dying. Vesicles originating from the cell membrane, which contain both chromosomal and plasmid DNA, have been found in 14 gram-negative species of bacteria. These "blisters" were able to transfer DNA to other bacteria in the environment (Dorward & Garon, 1990).

Many extraction methods exist to analyse for DNA in the environment. All told, larger amounts of DNA are extracted directly from the soil than can be achieved by extraction from the cells in the soil (Steffan et al., 1988), thus serving as direct evidence of the occurrence of free DNA. Investigations also exist which show that naked DNA molecules in soil originate from micro-organisms which are no longer present in the habitat (Spring et al., 1992), yet another indication that phenotypically and genetically dead are two quite different things in an ecological context.

In the few studies that have been undertaken (Paul & David, 1989: Lorenz & Wackernagel, 1994), the genotype, whether the organism concerned is a wild type or has been genetically engineered, has not had any influence on the liberation of DNA by the bacterial population. Liberation of DNA must be looked upon as a physiological process, but its extent may be significantly affected by abiotic (e.g. ionic strength, pH, temperature) and biological factors. Among the last mentioned are bacteriophages, which are both far more widespread and have a broader host spectrum than previously assumed (Børsheim, 1993), and protozoans (Turk *et al.*, 1992).

Bacteria in natural habitats are often starved, and many species are found in a living but not cultivable form (Kaprelyants *et al.*, 1993). Such cells preserve their genetic information. This was shown in non-cultivable *E. coli*, where a recombinant plasmid was stable after 28 days in an artificial sea-water microcosm (Byrd *et al.*, 1992). This illustrates that genetically modified, living, but non-cultivable bacteria can be sources of biologically active naked DNA in Nature when, often after long periods, the integrity of the cells is lost (Lorenz & Wackernagel, 1994).

Protection of naked DNA in Nature

Particles found in soil and sediment, such as quartz, feldspar and clay minerals, as well as those suspended in naturally occurring water, have the ability to bind both organic and inorganic material. When DNA is bound to some of these types of particles, it is protected from being broken down and must therefore be looked upon as a source for the transfer of genetic information (Lorenz & Wackernagel, 1994, Nielsen et al., 1998; Traavik, 1999). Parameters which influence the speed and scale of DNA binding are the type of mineral, the valency and concentration of cations and pH in the bulk phase, whereas the temperature, DNA conformation and size of molecules seem to have little or no effect (Lorenz & Wackernagel, 1994). Clays have up to 700 times higher binding activity than quartz sand. Adsorption of DNA to minerals takes place very rapidly, and when the complexes are first formed they are very stable. Increased concentrations of multivalent cations and low pH will increase the amount of adsorbed DNA (Romanowski *et al.*, 1991, 1993; Khanna & Stozky, 1992; Lorenz & Wackernagel, 1992).

Another important phenomenon, documented in many studies (reviewed by Lorenz & Wackernagel, 1994; Nielsen et al., 1998), is that adsorbed DNA is much more resistant to enzymatic breakdown than DNA dissolved in a liquid phase. 100 to 1000 times more DNase 1 or *Serratia marcescens* nuclease is required to break down adsorbed DNA than the same amount of DNA in solution.

Degradation of DNA in Nature

A rule of thumb would be that plasmid DNA in waste water will be completely broken down (converted from supercoiled helix to open circles or linear forms) within minutes. In fresh water and sea water, the same process takes hours, whereas DNA can persist intact for weeks, even months or years, in soil (Romanowski *et al.*, 1992, 1993) and marine sediments (Novitzky, 1986). Then, in addition, in all these cases, there is the unanswered question of whether open circles and linear plasmid and chromosomal DNA can have undesirable biological effects, too, since these forms are equally well taken up by competent cells and their genetic information can be activated by cellular processes (Lorenz & Wackernagel, 1994; Nielsen et al, 1998).

Uptake of nucleic acids in the mammalian organism

In many biological systems, it has been demonstrated that mammalian cells can take up foreign DNA in a manner that permits biological activity. This is, of course, precisely the basis for transfections in cell cultures, genetic modifications of plants and animals, for gene therapy and for DNA vaccination. However, are the epithelial surfaces in the gastrointestinal or respiratory tracts of the mammal impervious barriers to the uptake of introduced foreign DNA, or can such DNA penetrate into the organism from the extensive epithelial surfaces in the body?

The fate of nucleic acids in the gastrointestinal tract was studied in ruminants and rats in the 1970's and 1980's. The limited sensitivity of the methods available at that time meant that lack of discoveries could not exclude that biologically active DNA could both be taken up from the intestinal tract of the individual, and be dispersed to the surroundings in the faeces.

These questions were re-evaluated using new, much more sensitive methods (Schubbert *et al.*, 1994). Mice were pipette-fed with circular or linear double-stranded M13 bacteriophage DNA, or this was added to the feed pellets. Sensitive hybridisation methods and PCR were then used to identify M13 sequences in the faeces and blood.

The results showed that 2-4% of the introduced M13 DNA could be identified in the faeces and 0.01-0.1% in the blood, where the DNA was found in both the serum and the cell fraction. Separate fragments measuring up to 1692 bp out of the 7250 bp total size of the M13 genome were found up to 7 hours after uptake. No difference was found between circular and linear DNA.

In more recent work, the same research group demonstrated that ingested DNA under some circumstances may be taken up from the intestines of mice, inserted into chromosomes and vertically transmitted to offspring (Doerfler et al., 1997; Schubbert et al., 1997; Doerfler et al., 1998).

The authors assume that other types of DNA would behave in the same manner, but they add that this must be investigated experimentally. These observations raise several challenging questions.

The efficiency of the oral DNA vaccines indicates that there may be a normal process whereby large segments of intact DNA trapped in particles get from the gut, through PPs into the nuclei of APCs (Lowrie, 1998) and perhaps other cells in the organism (Schubbert et al., 1997; Doerfler et al., 1998).

To what extent can DNA which is taken up from the intestines be internalized by cells in various organ systems? Can foreign DNA in the blood stream of a pregnant female pass across the placenta and enter the foetus (Doerfler et al., 1998)? Can foreign DNA which is taken up from the intestine contribute to mutagenesis and oncogenesis? Can DNA which is released by way of the faeces be taken up by other organisms and can this have biological consequences? To what extent do the answers depend upon the DNA's sequence, structure and complex formation with proteins in the host organism, and pollution in the environment, etc.?

The genomes of the polyoma viruses (SV-40, BK virus, mouse polyoma, etc.) are small (ca. 5 kbp), circular, double-stranded DNA molecules which are able to function as expression

vectors in mammalian cell cultures. Transfection of cell cultures with naked, genomic polyoma virus DNA results in infection with production of virus particles. In a series of viral infection trials carried out at the Department of Virology in the University of Tromsø, one of the controls was naked genomic virus DNA injected intravenously into rabbits and mice. Based on what was known from the literature, and so-called conventional wisdom, it was assumed that DNA under such circumstances would be rapidly broken down by nucleases and, in practice, be devoid of biological activity. It was therefore most surprising, as well as being a lesson to us, that both viral genetic expression and full, productive viral infection were indeed initiated in the animals (Fredriksen, 1993; Fredriksen *et al*., 1994). Likewise, more recently efficient expression of intravenously delivered DNA in rat muscle was demonstrated (Budker et al., 1998). High levels of foreign gene expression was observed in the liver cells of rats, mice and dogs when naked plasmid DNA was injected into blood vessels supplying the liver (Zhang et al., 1997). Naked DNA was integrated into cellular chromosomes and expressed in human and pig skin (Hengge et al., 1995). Unexpected side effects such as myositis appeared when plasmids carrying the gene for a t-RNA activating enzyme was injected into the bloodstream of mice (Blechynden et al., 1997).

A number of recent reports have demonstrated that naked DNA may be taken up in unpredictable ways. The ability of naked DNA to penetrate intact skin has been known for years, e.g. within weeks of applying cloned DNA including a human oncogene to the skin on the back of mice, tumors developed in endothelial cells lining blood vessels (Brown, 1990). More recently gene therapy based on cutaneous application of DNA is seriously considered (Khavari, 1997).

That nucleic acids are taken up and have biological activity is obviously not a general phenomenon. Throughout the history of evolution, animals and people have been receiving foreign DNA from other animals and plants through uptake of nutrients and breathing of air. The problem is just, yet again, that we know that in the case of a few, perhaps rare, combinations of nucleic acids and circumstances, nucleic acids will be able to be taken up from the mucous membranes. However, we have no knowledge of the sequences, structures or environmental factors which can contribute to such stability. Nor can we therefore, at the present time, predict what type of DNA will avoid rapid breakdown in the organism and which environmental factors may contribute to this.

Nucleic acid receptors

Oligo- and polynucleotides cannot diffuse through the lipid membranes of living cells. In some eucaryotic cells, it has been shown that nucleic acids can be taken up by endocytosis which is mediated by nucleic acid-specific receptors (Vlasov et al., 1994), and similar mechanisms may be active in bacteria, too (Dreiseikelmann, 1994; Lorenz & Wackernagel, 1994). Following uptake, the nucleotides find a way of escaping from the endosomes in eucaryotic cells and reach nucleic acids that are located in both the cytoplasm and the nucleus (Vlasov *et al*., 1994). Bacteria normally remove the foreign DNA that was taken up using restriction enzymes which distinguish between their own and foreign DNA, but this mechanism can clearly also fail under certain circumstances (for recent review, see Nielsen et al., 1998; Traavik, 1999).

The biological and evolutionary importance of these mechanisms is not known and we have no knowledge about the difference between nucleic acids that are taken up in biologically active form and those that are broken down. Nor do we know whether environmental conditions can

increase or reduce the expression of the nucleic acid receptors, or whether this can affect the uptake and the further handling of nucleic acids in these cells.

What if DNA recombination takes place?
Major genomic re-arrangements, such as duplications, delesions, translocations or insertions (integrations), are important for evolution. Duplications give, for example, additional copies of genes, and these can accumulate mutations, thereby offering opportunities for further evolution. Translocations and delesions can fuse genes, thus creating proteins with new combinations of functional domains, or change the surroundings of one gene thereby helping it to be influenced by new regulatory mechanisms. Insertions of foreign DNA into a genome are important steps in horizontal gene transfer and help to overcome the need for repeated evolution of similar functions in different organisms. In the context of evolution, they may be looked upon as some of the positive effects of genomic re-arrangements.

However, genomic re-arrangements may also have serious damaging effects when they occur at the wrong places, at the wrong time, or on an abnormal scale (Doerfler et al., 1997. Re-arrangements may be the cause of cell growth aberrations, and the degree of re-arrangements often increase during the development of malignant tumors (Croce, 1987). They can cause the death of foetuses and developmental defects, metabolic illnesses and hereditary disorders such as Duchenne & Becker's muscular dystrophy (Bakker *et al*., 1987).

Such genomic re-arrangements may be the result of legitimate recombination which take place between long, homologous sequences (Anderson & Roth, 1977, 1981), or what are referred to as site-specific recombinations which are responsible for movements of specialised elements and genomic regions (e.g. transposons, mobile elements, etc.) However, they can also arise as a consequence of illegitimate recombinations between sequences with little or no homology (Ehrlich *et al*., 1993).

Illegitimate recombination is important because it is not confined to duplications or special, relatively rare sequences. Illegitimate recombination can therefore take place anywhere within a genome. Such recombination is probably universal, since it has been found in whichever organism has been searched for it, and it probably takes place far more frequently than has so far been imagined (Schrempf, 1985; Ehrlich *et al.*, 1993, Zuchman-Rossi et al., 1998; Dellaire and Chartrand, 1998; Gorbunova and Levy, 1997; Kusano et al., 1997; Clegg et al., 1997)).

The general mechanisms for initiating illegitimate recombination are not well known, but there are probably several of them. Deducing the mechanisms behind a proven, naturally occurring, illegitimate recombination is very difficult because the sequence of the primary genomes is usually unknown and the re-arranged genome is first recognised after many generations. This gives time for secondary re-arrangements to have occurred, a feature often seen in studies of gene amplifications (Smith *et al.*, 1990). To identify the various factors that affect illegitimate recombination and determine their significance it is therefore necessary to establish model systems. So far, such model systems have mostly been established in micro-organisms, but the aim is that these will provide concepts to explain phenomena in any type of cell (Ehrlich *et al.*, 1993). In spite of that, results are no accumulating to strongly indicate that illegitimate recombination is a major driving force in evolution of plants, animals and microorganisms, as well as a major cause of disease (Zuchman-Rossi et al., 1998; Dellaire and Chartrand, 1998; Gorbunova and Levy, 1997; Kusano et al., 1997; Clegg et al., 1997). In transgenic mice

(Dellaire and Chartrand, 1997) and in plants (Gorbunova and Levy, 1997) illegitimate recombination leads to the random and unpredictable integration of transgenes in the recipient chromosomes.

It is perfectly clear that a great deal remains to be learnt before we can claim more than a very rudimentary understanding of the phenomenon of illegitimate recombination, and still more before we can hope to control it (Ehrlich et al., 1993). Genomic re-arrangements are often found to modify, in undesirable places, gene constructions that are meant to be for biotechnological use (Ehrlich et al. 1986, 1993, Gorbunova and Levy, 1997). This should perhaps, itself, give grounds for warning signals about what may happen if such processes take place after GM constructions have established themselves in an ecosystem.

Horizontal gene transfer

For any given gene construct or GMO which is released, or escape, to the environment, the state of our present knowledge neither allows pre-assessment of probability nor consequences of horizontal gene transfer. Hence, according to the definition of risk, risk assessments become impossible at the moment. Only extensive research on the mechanisms of horizontal gene transfer and on ecosystem interconnections can change this situation.

Horizontal (lateral) gene transfer is defined as non-sexual transfer of genetic information between genomes. The expression is generally used about transfer between core genomes in different species, but it can also be applied to genetic transfer between different organs in the same or different species. Transfer with the aid of parasitic species or symbionts to host species can also be included.

Horizontal transfer is thus distinct from the ordinary form of gene transfer which takes place vertically from parent to offspring. There is now good evidence that horizontal transfer takes place for both genomic (usually non-mobile) sequences and sequences derived from transposable genetic elements or mobile introns. Documented cases exist of genomic sequences being transferred from eucaryotes to procaryotes, from procaryotes to eucaryotes, between procaryotes and between eucaryotes (reviews in Heinemann, 1991; Kidwell, 1993; Harding, 1996; Wöstemayer et al., 1997; Nielsen et al., 1998; Traavik, 1999).

The possibility that genetic information could move between distantly related species was an idea which met a great deal of opposition in traditional biological schools when it was first put forward a couple of decades ago. The opposition is understandable enough, because this concept, viewed superficially, conflicts with both explanatory models based on phylogenetic trees in taxonomy and with the important role of reproductive isolation as a mechanism for species formation. However, horizontal gene transfer is now attaining more and more support. Not only are there dozens of examples of probable horizontal transfer, but the molecular mechanisms which may contribute to such transfer are continually being observed, both physical means of transfer for DNA between cells and recombination mechanisms which can lead to the gene transfer becoming permanent. Horizontal transfer of genes is now an indisputable fact, and the most important question that remains is whether such transfer takes place at a speed that significantly affects evolution.

The debate has mainly concerned horizontal transfer of entire genes, but for *E. coli, Streptococcus* and *Neisseria* species it has been shown that far shorter elements are stably

transferred and can give rise to mosaic genes. There are strong indications that this takes place in eucaryotic organisms, too, exemplified by cytochrome c in plants and betaglobines in mammals.

Theoretically, shorter DNA sequences will, beyond our knowledge, be able to contain control elements for expression of genes (e.g. promotors or enhancers) which can change the amounts of some gene products in the recipient, perhaps with substantial biological consequences.

A general evolutionary theory which incorporates horizontal transfer of genes astride taxonomic boundaries seems to be able to give a satisfactory answer to the important question of : Why is the molecular biology of all living organisms so uniform? Despite species formation, biology has maintained a uniformity which even permits transgenic animals to be constructed in the laboratory.

Many evolutionists still believe that an evolutionary theory that incorporates horizontal transfer conflicts with the useful concepts, "phylogenetic trees", and "reproductive isolation". It is far simpler for molecular and cell biologists to accept it. However, the widespread horizontal transfers that have taken place for sub-populations of *E. coli* and *S. typhimurium* has not prevented these bacteria from being capable of being placed in phylogenetic, tree-like evolutionary models. Some microbiologists who studied plasmids had previously assumed that cross-species gene transfer took place, and they used this as the basis for arguing against the possibilities for a meaningful phylogenetic classification of bacteria. Possibly, they did not go too far (for review see Nielsen et al., 1998; Traavik, 1999).

We thus know that there are limitations as to what kind of DNA can be transferred, but we do not know what kinds of mechanisms which sort DNA for transfer and are therefore unable to pre-assess whether a plasmid or another genetic construction we make use of will be transferred horizontally, when it will transferred and where it will end up. Furthermore, as stated by Nielsen et al. (1998): "Transfer frequencies should not be confounded with the likelihood of environmental implications, since the frequency of horizontal gene transfer is probably only marginally important compared with the selective force acting on the outcome". We know very little about selective forces in different ecosystems.

For any given gene construct or GMO which is released, or escape, to the environment, the state of our present knowledge neither allows pre-assessment of probability nor consequences of horizontal gene transfer. Hence, according to the definition of risk (see chapter 2), risk assessments become impossible at the moment. Only extensive resarch on the mechanisms of horizontal gene transfer and on ecosystem interconnections can change this situation.

Even when the antigen expression from a plasmid is placed under the control of an eucaryotic promoter, conservation of regulatory elements between phyla can sometimes result in low levels of gene expression in unlikely circumstances (Lowrie, 1998), i.e. the nature of the actual DNA used may play a role. Furthermore, at this stage we simply do not know how plasmid DNA ever gets from a phagolysosomal vesicle into the nucleus of an APC, or any other cell type, in a functional state (Lowrie, 1998), i.e. again, the kind of DNA mayt play a role.

Evolution favours those organisms which have a suitable balance between genetic variation and genetic stability. However, we do not know how such a balance is established and maintained, and consequently neither whether it can be upset nor how that can happen.

Barriers to horizontal transfer
For horizontal transfer to take place, genetic material has to overcome at least two types of hypothetical barrier (Heinemann, 1991), an introduction barrier and an establishment barrier. These barriers ought to make contact between genetic donors and recipients difficult, degrade genetic material, exclude foreign material from replication and/or segregation processes, and prevent the expression of genes which are required for inheriting transferred molecules. It is clear that the introduction barriers are often broken and that a network for genetic exchange between organisms exists.

Many observations and experiments indicate that the introduction barriers are often broken so that DNA wanders between phylogenetically remote species. Many bacteria may be naturally competent for being exposed to transformation with DNA from any source whatsoever. Conjugational transfer of DNA does not only take place within species, but also across species boundaries, and even kingdoms. Agrobacteria may, for example, transfer DNA to their plant hosts, and effective conjugation can take place between *E. coli* and several yeast species. This, in turn, indicates that establishment barriers are very effective and that these are necessary for species to be able to remain distinct in a world of genetic promiscuity (Heinemann, 1991).

The problem is that we know that establishment barriers, too, may be broken, but we do not know the mechanism and can therefore not guard against such highly undesirable occurrences. We undertake modifications and mutations which are intended to make nucleic acids more effective in use. Examples have been published where small changes in a DNA sequence can change the host spectrum for a transferable genetic element (Kipling & Kearsey, 1990). Do we undertake, without being aware of it, such changes with our genetic constructions and modifications? Are the barriers capable of being influenced by the amounts of naked DNA, and how much DNA is required in a given situation to break down an ecological barrier? Finally, it is also in this context important to remember that the plasmids used for immunisation and gene therapy are both procaryotic and eucaryotic shuttle vectors. Consequently, if they escape into Nature they can multiply in and disperse with representatives of both kingdoms.

General mechanisms for horizontal gene transfer
The occurrence, and mechanisms for, horizontal (lateral) transfer of genes have been remarkably little studied, especially in eucaryotic cells and organisms. However, there are some brief and useful reviews of the topic (Heinemann, 1991; Landman, 1991; Bogosian & Kane, 1991; Powers *et al.*, 1991, Thakur *et al.*, 1991; Kidwell, 1993; Lambowitz & Belfort, 1993; Dreiseikelmann, 1994; Capy *et al.*, 1994; Lorenz & Wackernagel, 1994; Harding, 1996; Wöstemayer et al., 1997; Nielsen et al., 1998; Traavik, 1999).

5.5 RNA vaccines

Although the RNA-based vaccination method appears to be efficacious, conclusions related to safety may be overstated, it is claimed (Dubensky et al., 1998). For instance, “infectious” RNA vaccines avoid the major drawback of true live virus vaccines, namely the possibility of

reversion to a virulent phenotype during passage in cell culture. But reversion to the virulent phenotype in the vaccinated recipient still remains a concern for RNA vaccines.

Also, this present author would like to add, recombination between related RNA molecules is a real concern. If an individual (human or animal) receives a naked RNA vaccine, intentionally or not, and is naturally infected with a wild type virus, hybrid viruses with unpredictable biological and pathogenic characteristics may result (see section 5.3).

Although in the laboratory, a main problem with RNA work is to avoid degradation due to ubiquitous RNases, it may be surprisingly resistent under natural conditions. Naked RNA can be identified for up to 2 days in unfiltered and 28 days in filter-sterilised sea water under experimental conditions (Tsai *et al.*, 1995), and has also proved to have an amazingly long survival time in soil (Greaves & Wilson, 1970).

The delivery of the TBE vaccine RNA by GeneGun bombardments was very effiicient (Mandl et al., 1998), requiring less than 1 ng of RNA. A rather surprising observation was the high stability of RNA coated on to gold microcarriers under standard 4 C storage conditions. Storage for up to 5 weeks (longest storage used) gave no detectable loss of infectious RNA activity.

RNA recombination

Most viruses have RNA genomes. Owing to the high error rate of RNA-dependent replication, RNA viruses exist as heterogeneous populations of molecules known as quasispecies (Domingo et al., 1996). The advantage of this to the virus species is that, as selection pressures change, a fit genome might already exist or can evolve rapidly. The disadvantage of the high replication error rate is that accumulation of too many mutations is damaging. However, genetic defects may be rescued by recombination with other molecules in a quasispecies that have functional genes (Lai, 1996). Furthermore, by RNA recombination, a virus can suddenly acquire entirely new traits, i.e. whole new genes, in one step.

Recombination is common in RNA viruses, and might be more importnt than accumulation of point mutations for significant evolutionary change and speciation of such viruses (Lai, 1996; Simon and Bujarski, 1994; review by Miller and Koev, 1998). Viruses in taxonomical groupings as diverse as the alphaviruses, coronaviruses and luteoviruses contain genes closely related to those of other groups, indicating recent recombination events in their evolution.

Understanding viral RNA recombination is most obviously important for the safe deployment of all kinds of vaccines which are genetically self-expressing, i.e. all kinds of "live" vaccines, edible vaccines, DNA and RNA vaccines. It is obvious that from a risk assessment point of view, this ought to be a key task that should have clarified before transgenic crops were taken into general use

RNA virus recombination events have been classified into homologous, aberrant homologous and non-homologous (Lai, 1996). However, it has more recently been proposed to revise the nomenclature into sequence similarity-essential, similarity-assisted and similariry-nonessential recombination (Nagy and Simon, 1997).

The mechanisms for RNA recombination are not known in all observed instances, but most evidence support a copy-choice process (Nagy and Simon, 1997; Nagy et al., 1998). This

implies that a RNA replicase switches from copying a RNA donor template to an acceptor template without releasing the nascent RNA strand, resulting in a hybrid RNA molecule. It is conceivable, though not proven, that more than two different RNA molecules might be involved in such a process, assuming that they were present in the same cell at the same time.

The implications of the RNA recombination concept for use of self-expressing vaccines ought to be self-explaining. If an RNA replicase and one foreign RNA species are present in a cell or organism, totally new species of viral RNA or mRNA may arise.

5.6 “Edible” vaccines

Edible vaccines are produced by genetically modified plants (GMPs). Little is known about the consequences of releasing genetically engineered plants into the environment. Recenttly it was demonstrated that transgenic plants may alter their biological environment, more precisely the root-associated bacterial populations (Oger at al., 1997). The ecological alteratins were both transgene-specific and target population-specific. The authors concluded that assessment studies on the introduction of a given transgene into a GMP will only be valid for the given transgene.

The unpredictability with regard to variability in expression levels for transgenes in plants is an acknowledged problem (Mor et al., 1998). Such variation is observed between different plants of the same transgenic line, and sometimes even within the same plant. Appreciable variation in transgenic expression levels has for instance been demonstrated between different tubers of the same potato plant.

The most serious scientifically based arguments against large scale, commercial use of the first generation GMPs, and hence also “edible vaccines” are based on the unpredictability with regard to *where* in the recipient cell chromosomes insertion of vector DNA takes place. The consequences of insertion may vary considerably according to the precise insertional location (Doerfler et al, 1997). This is valid for the expression of the inserted transgene as well as for changes in the recipient organisms own genes and their expression levels.

Some of the most prominent uncertainties are related to the fact that the recipient organism has received a new promoter/enhancer. These elements are governing the gene expression levels of their attached transgenes, but after insertion they may also change the gene expression and methylation patterns in the recipient chromosome(s) over long distances up- and downstream from the insertion site. Promoter/enhancers function in response to signals received from the internal or external environment of the organism. For a GMO this results in unpredictability with regard to:

- The expression level of the inserted foreign gene(s).
- Expression of a vast number of the organism’s own genes.
- Influence of geographical, climatic, chemical (i.e. xenobiotics) and ecological changes in the environment.
- Transfer of vector sequences within the chromosomes of the organism and vertical and/or horizontal gene transfer to other organisms.

Genetic pollution from GMPs is a real option. This can be exerted by cross-pollination, unplanned breeding and horizontal gene transfer (reviews: Kidwell, 1994; Nielsen et al., 1998;

Traavik, 1999) Such events may result in extensive and unpredictable health-, environmental and socioeconomic problems. Environmental persistence and transfer of nucleic acids are extensively discussed in the two latter references. The issue has an added reality after the demonstrations by a highly respected research group that ingested DNA under some circumstances may be taken up from the intestines of mice, inserted into chromosomes and vertically transmitted to offspring (Doerfler et al., 1997; Schubbert et al., 1997; Doerfler et al., 1998).

A number of unpredicted incidents have already taken place with GMPs. They have been extensively reviewed in recent publications (Ho, 1998: Ho et al., 1998; Myhr and Traavik, 1999: Traavik, 1999). Just a few examples are cited here:

Recently it was demonstrated that self-pollinating GM plants may have a forced, augmented capability to cross-pollinate other plants, with a resulting transfer of inserted transgenes (Bergelson et al., 1998). The unpredictabilty was demonstrated by the fact that inbred, identical plants genetically modified in separate experiments had differing abilities to crosspollinate other plants. Although the experiments were carried out on a single plant species, Arabidopsis thaliana, these results have general interest, also because the inserted gene (csr-1) have been introduced in various plant species as an alternative selection marker to replace antibiotic resistance genes.

Researchers at the Scottish Crop Research Institute in Dundee have demonstrated indirect ecological effects of GM potato plants. The plants expressed an inserted lecthin gene in order to reduce aphid attacks. Ladybirds predating lecthin containing aphids had their life time expectancies and reproducibility significantly reduced. Likewise, researchers at the Swiss Federal Research station for Agroecology in Zürich have demonstrated serious harm to lacewings foraging on aphids affected by the insecticide Bt toxin produced by GM maize (Williams, 1998). It is already a major world-vide agricultural problem that natural predators of crop-ruining insects disappear. An acceleration of this process would be tragic.

Field trials in Denmark and Scotland have shown that GM oilseed rape may transfer their inserted transgene by crosspollination of wild relatives (Mikkelsen et al., 1996), while experiments in France have demonstrated transfer of resistance genes from rape to radish (Chévre et al., 1997). Similar examples, with spread of transgenes over long distances, have been demonstrated for other GM plant species. Organic plant farmers in European countries have initiated legal actions on this background. When their farms are situated in the vicinity of GM crop fields, their products may be deprived of the "organic" labeling.

6 Special considerations: Ecosystem and species

6.1 Xenobiotics

Very little work seems to have been done with regard to how xenobiotics may interfer with horizontal gene transfer under natural conditions or in microcosm and other types of controlled experiments. However, a vast literature concerning other effects of environmental pollutants indicate that such effects may exist (i.e. Ferguson, 1998; Smital and Kurelec, 1998; Wirgin and Waldman, 1998; Steinmetz et al., 1998; Tyler et al., 1998; Darbree, 1998; Williams et al., 1998; Zacharewski, 1998).

Xenobiotics are, literally, compounds that are alien in the biosphere. Nevertheless, with such a narrow definition, metals, some pesticides and many organic chemicals would not be considered xenobiotics because they are also found naturally in ecosystems. The definition does not take human activity into account, which may increase the concentration of natural compounds to levels which give damaging effects. The essential element phosphorous is a good example. It is not usually a xenobiotic compound, but in large amounts we know it can create major environmental problems. Consequently, the following definition is used: xenobiotics are compounds which people release into Nature in concentrations that create undesirable impacts.

Different xenobiotics have properties and biological activities that enable us to envisage at least two different sets of possible impacts on the fate of naked DNA in an ecosystem.

Some xenobiotics can act as mutagenes (this applies to both radioactive substances, polluting industrial chemicals and plant protectants). Mutagenes can result in naked DNA that escapes or is released having its sequence or structure changed. This, in turn, can affect the possibilities for DNA uptake in cells and organisms, horizontal transfer and long-term establishment in the ecosystems in ways which are totally unpredictable for us. Kipling & Kearsey (1990) have reported examples of minor changes in a DNA sequence altering the host spectrum of a transferable genetic element.

Some xenobiotics can affect cell membrane and/or intracellular functions in ways which can very well be thought to influence the ability of cells to take up and horizontally transfer naked DNA. This concerns the structure of cell membranes and the content of both surface receptors and transport canals, and also for intracellular signal conversion and gene expression. For instance, xenobiotics which mimic hormones or affect the local conditions in the organ systems of mammals (e.g. respiratory passages) may change the possibilities for both uptake and establishment of foreign nucleic acids in animals and people.

Some xenobiotics will be found in both categories, and we do not know how the sum of the impacts of such substances will turn out. Likewise, up to several individual compounds from each category will often pollute the same environment. We have no knowledge of how such situations affect DNA uptake and dispersal in the ecosystems.

Many of the xenobiotics with which man has polluted his environment during the past decades (e.g. herbicides, pesticides, heavy metals, emissions from industry and burning of fossil hydrocarbons, etc.) have in common that they are chemically inert and hydrophobic. *Hydrophobism* means that they easily enter organisms by diffusing through biological membranes, are difficult to separate in urine and gall, and accumulate in certain areas of the cell, including the phosphorous-lipid double layer in the membranes where they are able to disturb normal cellular functions (Lundgren & DePierre, 1990).

Most organisms have natural inactivation mechanisms for xenobiotics, but the efficiency in mammals may, on a genetic basis, vary several hundred times between separate individuals of the same species (Lundgren & DePierre, 1990). The biochemical processes between closely related species of fish may also vary so much that one species develops liver cancer through a concentration of polycyclic aromatic hydrocarbons which do not affect the other species (Stein *et al.*, 1990). A number of intermediaries from the breakdown of xenobiotics which are not inactivated sufficiently quickly may attach covalently to both RNA, DNA and proteins, and such attachment may lead to both toxic and teratogenic effects, carcinogenesis and mutagenesis (Lundgren & DePierre, 1990; el-Bayoumy *et al.*, 1994).

Xenobiotics are obviously, in practice, never found one at a time in the environment. Interactions between several xenobiotics will probably affect organisms and cells differently from a single substance alone (Hicks *et al.*, 1990). It is therefore surprising that relatively few studies are found which take this into account. Whereas antagonistic effects between different contaminating chemicals have been investigated for many years, it is first quite recently that synergistic effects have been studied. However, a number of articles in the last few years have demonstrated that the synergistic effects are unpredictable. For instance, severe mutagenic synergism has been proved between different kinds of pesticides, between pesticides and X-rays, between heavy metals and radioactivity, etc. (Lee *et al.*, 1994; Shima & Ichikawa, 1994; Newman *et al.*, 1995).

Synergism may mean that far lower concentrations of the individual xenobiotics have biological activity and the absolute minimum values vary according to which other forms of contamination are found. It may also mean that even the phenotypical evidence or symptoms at low concentrations of individual xenobiotics are changed. This is an enormous field of research which, for the moment, is almost untouched in any context, and not least as regards the ecological risks of release of recombinant DNA.

Data bases contain good review articles about the ability of micro-organisms to break down xenobiotics, and how the life processes of micro-organisms are affected by some xenobiotics (see, for example, Ghiorse & Wilson, 1988; Cork & Krueger, 1991; Stotzky *et al.*, 1993). However, it is difficult to find references concerning how xenobiotics affect the competence of organisms for uptake of naked DNA, the permissiveness for viruses, the ability for conjugation, etc.). This is curious, because the types of cellular functions that are usually affected by xenobiotics, such as the composition and permeability of cell membranes, the

synthesis of nucleic acids and proteins, etc., can very well be envisaged to have an impact on the possibility for horizontal transfer of genes. The extent to which xenobiotics affect living cells and organisms depends upon the specific physico-chemical conditions, such as the type of soil, the temperature, the water content and the pH, factors which, in turn, may be affected by other types of contamination, local emissions, etc. (Hicks *et al.*, 1990).

The various methods employed to make bacteria competent for genetic transformation under laboratory conditions can be divided into the following main groups (Mercenier & Chassy, 1988): i) treatment with solutions of calcium chloride or chlorides of other elements, including magnesium, barium, rubidium, strontium and mixtures of such heavy metals; ii) treatment with EDTA or other chelating aids; iii) treatment with enzymes (e.g. muraminidases or proteases); iv) fusion of cells with DNA, with other cells, or with DNA packed in liposomes; v) freezing and thawing of cells; vi) exposure of cells to electric fields; vii) bombardment of cells with small particles which transport DNA into cytoplasm (biolistic transformation).

It is not difficult to imagine that bacteria in the ecosystems can be exposed to conditions which are concurrent with the laboratory conditions listed above, and to up to several at a time. The concentrations of heavy metals may vary within quite wide extremes over time and from place to place. The same applies to phosphate emissions, and it is known from laboratory experiments that enhanced phosphate concentration may reduce the thickness of the capsules of some bacteria, thereby increasing the competence for DNA uptake (Page, 1985). How variations in pH, for instance in the form of acid precipitation, will affect the uptake of naked DNA in micro-organisms, plants and animals is in reality completely unknown.

A large number, and large amounts, of chemicals which mimic or interrupt mechanisms of hormones have been released into the environment since the Second World War. If vertebrates are exposed in the foetal state or after birth, many of these chemicals can disturb the development of the important glandular systems (endocrinal organs) of the body, and thereby of the other organs that are dependent upon correctly tuned hormone signals for normal development and function. Such effects on single individuals are permanent and irreversible. Effects which span over generations can arise as a consequence of the exposure of a female to chemicals at any moment in her life before she produces offspring. This is due to the storage of the hormone-imitating chemicals in her body fat. These are mobilised during egg laying or pregnancy. More than 50 chemicals that are widely dispersed over the entire globe, herbicides, fungicides, insecticides, nematocides, industrial chemicals such as dioxin, PCB and phenols, as well as metals such as cadmium, lead and mercury, can act as hormone imitators (Colborn *et al.*, 1994).

On the cellular level, hormone-imitating xenobiotics will be capable of disturbing the transfer of signals from the cell surface to the nucleus, and hence the genetic expression of the cell. We know nothing about how this affects the opportunities for foreign DNA to be taken up and few of the biological consequences in eucaryotic cells and organisms.

One of the most cunning threats to which the environment and public health are now exposed to is the group of chlorinated hydrocarbons which go under the collective name of *dioxins*. This group includes dioxin proper, TCDD (2,3,7,8 tetrachlorodibenzo-*p*-dioxin, and the closely related compounds CDD (chlorinated dibenzodioxins), CDF (chlorinated dibenzofurans) and PCB (polychlorinated biphenyls).

The great biological potential and the fundamental level in living cells which the dioxins act upon are analogous to many well-studied steroid hormones. However, the individual dioxins can either enhance the effect of naturally occurring hormones or counteract them. The dioxins have the ability of changing the growth pattern and differentiation programmes of a large number of target cells by initiating biochemical and biological processes which may give a whole range of responses in animals and humans (U.S. Environmental Protection Agency, 1994a).

The presence of the dioxins in the environment is quite obviously, and without doubt, due to man-made pollution. Incinerating plants and industrial emissions are the greatest sources. The primary mechanism for the entry of the dioxins into food chains is precipitation from the atmosphere onto plants and the soil. Humans are exposed through intake of food containing small amounts of dioxins. The most important sources are fatty dairy products, fish and meat (U.S. Environmental Protection Agency, 1994b, 1994c).

A series of common biological steps have been identified and described that are preconditions for most, perhaps all, of the effects of dioxins observed on vertebrates, including man. The first step is the binding of dioxin to the intracellular protein called the Ah receptor. This receptor is a gene regulating protein which displays many similarities common with steroid hormone receptors. Activation of the receptor is a two-stage process that comprises the binding of dioxin and dissociating of hsp90 from the receptor protein. When the receptor is activated in this manner it can attach itself to XRE (xenobiotic response element) sequences in cellular promotors and enhancers, and alter the genetic expression of cellular genes (Wilhelmson *et al.*, 1990).

When one is working with a receptor model, where xenobiotics imitate or interfere with natural compounds in living organisms, the effect can give several endpoints. Bimodal responses can be observed, depending upon the length of exposure, age and sex. In the earliest DDT trials, high doses were used and, for example, thinner egg shells were not found in bird species whereas the natural effects on them later proved to be catastrophic. Important impacts can thus be overlooked when high-dosage tests are used. In the short term, the effects concerned may be subtle and occur in the form of altered or reduced functions, whereas dramatic changes will not be seen which increase mortality or serious malformations. Dioxin-mediated changes of genetic expression may have several other consequences that have been recognised in animals for many years, and the available literature strongly indicates that people respond in the same way. A large number of foetus-developing programmes can be disturbed. This has been proved for several species within three classes of vertebrates. Negative impact on the reproductive ability of both masculine and feminine individuals is well documented, and there is clear connection between exposure to dioxins and increased cancer mortality. Many of the functional disturbances, especially with regard to impact on foetuses, occur with nanogram and picogram concentrations, just as in the case of natural hormones (Colborn, 1994; Environmental Impact Assessment Review Team, 1994). The same is the case for effects which influence sexual traits and fertility. The impact of such properties may already be in process of attaining epidemic proportions in animals and people without us having so far acknowledged it (Colburn, 1994, 1995a,b; Colborn et al., 1998; Gray et al., 1997)). There is a continuum of responses on the exposure of organisms to dioxin-like chemicals. As the total load increases, the probability for individual impacts and the degree of collective effects increase. On the basis

of such a continuum, we have opportunities for acknowledging the link between early effects which are necessarily realised as damaging, and late effects which definitely are damaging (U.S. Environmental Protection Agency, 1994a, b, c).

6.2 Species

Fish

The growing demand for fish and shellfish products in human and animal consumption, combined with the continuous decline of wild fishery resources (Kaushik, 1997), have contributed to make aquaculture the fastest growing segment of agriculture in many parts of the world (Hanfman, 1993). As with other intensive farming operations, however, this rapid growth has been accompanied by a proliferation of profit-limiting infectious diseases. Pathogens now constitute the most important cause of economic loss for fish and shellfish farmers (Meyer, 1991), destroying 10% of all cultured animals (Leong and Fryer, 1993).

Chemicals and antibiotics can be used to control most bacterial and parasitic diseases, but not viral infections. However, even when these treatments are effective, vaccination appears a better alternative because of environmental concerns. Development of commercial vaccines against several bacterial diseases was relatively easy since they were made of inactivated bacteria (Newman, 1993). For viral and parasitic diseases, vaccine development has proven more difficult. Whole killed, live attenuated and subunit vaccines have worked to some extent, but they all have particular safety, environmental or economic disadvantages. Only two viral vaccines for fish have been commercialized. One of these is against IPN virus, made of recombinant VP2 protein incorporated into a multivalent vaccine by Intervet Norbio, Bergen, Norway.

The potential environmental problems connected with use of live vector-vaccines and DNA/RNA vaccines in aquaculture are obviously even more serious than in terrestrial ecosystems. Particles, microorganisms, viruses and DNA may be distributed over vast areas, distances and phylae, due to the relative lack of physical and physiological barriers. Hence, live attenuated or genetically engineered vector vaccines have not been introduced, or seriously considered. However, various investigations to adapt DNA vectors to fish vaccinology have been performed.

Relatively little is known about the expression of foreign genes in fish. A few studies have shown strong expression of reporter genes in fish injected with plasmid DNA (Hansen et al., 1991; Rahman and MacLean, 1992; Gómez-Chiarri et al.,1996), and very recently protective immunity following DNA vaccine injection in fish has been demonstrated (Anderson et al., 1996; Lorenzen et al., 1998; Heppell et al., 1998). Both antibody and cell-mediated responses were recorded.

It is now seriously suggested to encourage the development of DNA vaccines for infectious fish diseases where traditional strategies have been unsuccessful (Heppell et al., 1998). Such vaccines would carry various attractive advantages: low cost; ease of production and quality control; heat stability; identical production process for different vaccines; the possibility to make multivalent vaccines by plasmid cocktails; intramuscular (i.m.) injections rather than

intraperitoneal, which facilitate use of fully automated devices and avoid some growth-retarding pathological changes (Poppe and Breck, 1997).

In view of the general characteristics of the aquatic ecosystems as well as the DNA vaccines, and the general lack of knowledge about their environmental destiny, non-target effects, horizontal transfer and influence on consumers; it is strongly advised against further application and commercialization of DNA vaccines for fish. A number of basic questions should be attacked first. After i.m. DNA plasmid injections in fish, reporter gene expression was detected in gills (Heppell et al., 1998). It was unclear whether this was due to primary DNA transfection of migrating cells passing through the injected muscle, or diffusion of injected DNA and secondary transfection of cells at distant sites. Whatever the mechanism, however, this indicates a route for potential exposure of the environment to genetically engineered DNA. The high levels of reporter gene expression over prolonged periods are adding to this potential environmental hazard, and are also worrying from a consumer's point of view.

7 Concluding remarks and recommendations

The title of this report provides its final and most clear-cut conclusion: from an ecological and environmental point of view many first generation live, genetically engineered vaccines are inherently unpredictable, possibly dangerous, and should not be taken into wide-spread use until a number of putative problems have been clarified. It is not possible for the moment to neither assess nor manage the environmental risks involved. Most probably we have not even conceived all theoretical risks at the present time. Taking the precautionary principle and sustainable development into consideration, it seems obvious that many live, genetically engineered vaccine strategies should not be rushed into common use within medicine, veterinary medicine or fish farming.

At the same time, the whole vaccine field appears as the Devil's alternatives: there must be serious, scientifically proven risks in order not to save lives and food resources with whatever means are available. For quite understandable, ethically and morally honorable reasons, this is a field where long-term, theoretical problems will tend to yield for dramatic short-term goals.

Technology is developed to achieve benefits and there are many tragic examples of how people, elated over these, have both overlooked and neglected to adequately investigate the possibilities for dramatic disadvantages, which have therefore first been acknowledged much later.

Frightening examples from the last half of the 20th century are the application of organochlorines and other chemicals to fight plant pests, and the "peaceful" exploitation of nuclear power. We are now aware that the environment on the Earth has been seriously damaged by these senseless encroachments on the ecosystems, but it will still be a long time before we are able to recognise how serious the damage is.

In both these cases, sectors of informed public opinion in many countries posed serious questions concerning the safety and possible side effects of their use. The research communities on the other hand, with a few brave exceptions, made themselves available for a naïve, optimistic development, and were unanimous in their view that there were no real risks of undesirable effects for health and the environment. The same experts and research milieus that had participated in developing the new technology were employed as advisors by political authorities in connection with the pre-assessment of risks and the setting-up of systems to record damage.

Researchers are people like everyone else. The ability for critical and objective evaluation of risks associated with a person's own lifework is not a predominant part of human nature. There was, and still is, a lack of competent, independent expertise in many technological fields.

Recent years have witnessed many examples of unforeseen side effects from "safe technology" having led to health risks and threatened to disturb the ecological balance.

Dogmas concerning absence of hazards have often been proven wrong (e.g. Titanic). A relevant example is the belief that DNA in food and forage can not be taken up from the

gastrointestinal tract. Some experimental studies, and the whole evolutionary history as well as our daily intake of vast amounts of DNA from various sources supported this belief. Absolute biological and ecological truths are, however, very rare, and rare phenomena may have important consequences when they take place.

Recently this was illustrated by the demonstration that following ingestion by mice, DNA from the M13 bacteriophage could be detected as relatively long fragments in faces, peripheral leukocytes, spleen- and liver cells in significant time intervals after feeding. In the cells the ingested M13 DNA was found in a chromosome-integrated form (Schubbert et al., 1997; Doerfler et al., 1997). When such DNA was fed to pregnant mice, the test DNA was detected in various organs from foetuses and new born animals (Doerfler and Schubbert, 1998). The experimental conditions strongly indicated that the DNA had been transferred across the placenta. The authors concluded that the consequences of foreign-DNA uptake in the context of mutagenesis and oncogenesis should be subject to controlled experiments. Such experiments are still absent. Another unclarified issue is connected with the detection of long M13 DNA fragments in the faeces (Schubbert et al., 1997). If enteric bacteria, unwanted establishment of sequences, take up such fragments from transgenes, i.e. antibiotic resistance genes, may take place in pathogenic or opportunistic bacteria.

Development of bacteria that are resistant to antibiotics now represents a brewing catastrophe. Multiresistant bacteria do not remain in hospitals but have now been spread to the "healthy" community and, moreover, to large numbers of freely living, naturally occurring species of bacteria (Davies 1994; Kruse, 1994: Kruse & Sørum, 1994; Thomson *et al*., 1994). Antibiotics have saved numerous human lives, prevented suffering and preserved food resources and valuable resources in animal husbandry and aquaculture. However, senseless use of antibiotics has at the same time resulted in microbes now being on the warpath. Strains of increasing numbers of microbe species that are important for medicine and veterinary medicine are being found to be resistant to all relevant antibiotics. "Old" infectious diseases, such as tuberculosis, are returning, and freely living bacteria in the ecosystems have acquired resistance to antibiotics. During the last few decades, confidence in antibiotics has, moreover, led to the stagnation of research and testing of alternative strategies for preventing and treating infectious diseases. These fields of research now have to be re-awakened, because no one, including the pharmaceutical industry, believes that the constant development of new antibiotics is the right path to take. Horizontal gene transfer of antibiotic resistance genes lies at the root of the problem.

Another striking example is represented by the BSE (Bovine spongiform encephalopathia) story. Against the explicit conclusions of experts, the BSE prions crossed the hypothesized "species" barrier and initiated new variant Creutzfeld-Jacob disease (nv CJD) in human beings. Recently it has been demonstrated that a vast number of BSE prion-carrying, symptom-free cattle may have been consumed, and at the moment the extent of nv CJD is impossible to forecast.

In these cases, and many others, the experts were wrong. To the extent that any prior investigations of damaging effects had been undertaken, methods and approaches had been used that were only capable of disclosing short-term effects, whereas in ecological contexts it is the long-term impacts that are most important and most serious. Long-term impacts in these contexts, and also in connection with the possible damaging effects of the dispersal of

genetically engineered vaccines means not months or years, but at least ten to hundreds of years.

"Technology" is derived from the Greek term "tekhne" which is connected to handicraft or arts. Our associations with the word include predictability, control, and reproducibility. The parts of genetic engineering that concerns construction of vectors are truly technology. But present time techniques for moving new genes into cells and organisms mean:

- No possibility to target the vector/transgene to specific sites within the recipient genomes. In practical terms this means that modifications performed with identical recipients and vector gene constructs under the same standardized conditions may result in highly different GMOs depending on where the transgenes become inserted.

- No control with changes in gene expression patterns for the inserted or the endogenous genes of the GMO.

- No control of whether the inserted transgene(s), or parts thereof, move within or from the recipient genome, or where transferred DNA sequences end up in the ecosystems.

In the light of this, it seems both pertinent and relevant to ask the question whether genetic engineering at its present level of development deserves the label "technology" at all.

While working with this manuscript it has become an established and deplorable fact to me that the definition of "safety" in vaccinology is very narrow and exclusive compared to the putative risks and hazards vaccine use may imply. Primarily, "safety" research is occupied with prospects of unintended and unwanted side-effects with regard to the vaccinees themselves. Secondly, such research may be directed towards non-target effects on unvaccinated individuals within the same species. Very small efforts have been dedicated to unintended and non-target effects across species- and biologic kingdom-borders. As illustrated by the processes and examples presented in Chapters 5 and 6, this narrowing of conception as well as intellectual and research strategies may leave many potential hazards and harms related to various vaccine categories unapprised, until one or more of them actually happen. Very few research reports concerning environmental or ecological effects of genetically engineered vaccines were published as late as January 1999. On the other hand, examples of scientists defending the total innocuousness of vaccines, without taking environmental and non-target effects into consideration, are numerous. Many seem totally religious in their belief, and prescribe strategies to convert the ignorant public and politicians (Danner, 1997).

Furthermore, I suspect that the lack of holistic and ecological thinking (Ho, 1998; Ho et al., 1998; Holdrege, 1998; Traavik, 1999) with regard to vaccine risks, is symptomatic for the real lack of touch between medicine and molecular biology on one side, and potential ecological and environmental effects of these activities on the other. A frightening small number of original research reports concerning environmental or ecological consequences of molecular biology applications or genetic engineering were published until January 1999 (Anonymus, 1997; Dobson, 1997; Myhr and Traavik, 1999; Traavik, 1999). I believe that we are here dealing with a void in medical education and cooperation focus, as well as a dangerous lack of focused research efforts.

Genetically engineered self-replicating and/or -expressing vaccines may turn out to be good equipment in science, but too dangerous for practical large-scale use. I find it evident that the various putative risk factors and hazards related to these vaccines ought to be adequately investigated before we and the ecosystems are massively exposed to them. Many of the vaccine constructs may have obvious value within basic and applied research, but should be kept contained until credible ecological risk assessments are possible. Such clarification will demand carefully planned investigations and adequately designed model systems for experimental research. In addition to basic knowledge directly applicable to risk assessments, enhanced insight into and awareness of general biologic and ecological interactions ranging from the molecular to the ecosystem level would be gained.

There are no controversies connected to the fact that subunit or peptide vaccines are the inherently safest alternatives with regard to unintended side effects, as well as unpredictable non-target effects. Such vaccines are also, beyond reasonable doubt, the potentially safest from an ecological and environmental point of view. The intensive search for alternative vaccine strategies have been motivated by the disappointing immune responses that have often been obtained, in particular with regard to CTL responses and mucosal immunity, by the use of subunit vaccines. But this situation may change dramatically during the near future. New insight into basic immunological mechanisms, new delivery systems as well as targeted stimulation and weakening of specific immunological responses will certainly contribute to this end.

In order to make reliable risk assessments and perform sensible risk management with regard to genetic engineering in general, and genetically engineered vaccines in particular, much pertinent knowledge is lacking. The prerequisite for obtaining such knowledge is science and scientists dedicated to relevant projects and research areas. In my mind it is the responsibility of the national governments and international authorities to make funding available for such research. On one hand, this is obviously not the responsibility of producers and manufacturers. On the other hand, risk-associated research must be publicly funded in order to keep it totally independent, which is an absolute necessity for such activities.

Finally, it must always be kept in mind that although vaccinology is the “Holy Grail” of medicine, there are other ways of preventing infectious diseases in humans and animals that must not be ignored. Many of the most burdening infectious agents of mankind and its domesticated animals are caused by pathogens that have reservoirs and are circulating among wildlife animals. By increasing our knowledge about these reservoirs, their occurrence, the transmission routes within and out of the indigenous ecosystems, we might be able to break transmission chains or keep our activities out of dangerous ecosystems. There is a void in knowledge about the ecological interactions for many important pathogens. This field is to some extent subdued by the confidence in vaccines, and hence another scientific orphan.

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Directorate for Nature Management

The Directorate for Nature Management (DN) was established in 1985, as a department under the Norwegian Ministry of Environment.

The Directorate is authorized to manage Norwegian nature through various laws and regulations. The DN is also responsible for identifying, preventing, and solving environmental problems, through cooperation, advice, and information to other authorities and public groups.

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