REVIEW

Targeting the mucosa: genetically engineered vaccines and mucosal immune responses

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The discovery that inoculation of DNA leads to strong and long lasting immune responses generated enthusiasm to assess the efficacy of various genetically engineered vaccines against mucosally acquired infections. Various techniques have been used to generate the most suitable DNA vaccines, ranging from immunization with naked DNA to utilizing genetically engineered recombinant viruses and bacteria to deliver the DNA. Different DNA vaccine modalities and mucosal immune responses to them have been discussed. It has been shown that even though intramuscular and intradermal immunization with these vaccines generates strong systemic responses, mucosal responses are not induced. It has been proposed that the site of immunization determines mucosal immune responses and that primed lymphocytes preferentially accumulate at sites where they have been induced thus generating the strongest cellular and antibody responses at the site of vaccination. The impact of the site of induction on mucosal immune responses to vaccines is discussed. It is possible to enhance desired vaccine effects in the mucosa and to modify the undesirable side effects. Cytokines such as IL-2, IL-12, IL-15 and IL-18 have been used to enhance CTL activity while IL-5, IL-6 and the chemokine MIP-1 α have shown the capacity to increase IgA responses to vaccines. Genes and Immunity (2000) 1, 308–315.

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The mucosal immune network: importance of the site of antigen presentation

Next to the skin, human mucosa is the largest organ of the human body that is continuously exposed to outside antigens, microorganisms and viruses. It represents an area of 400 m² that is well equipped to deal with daily insults by infectious agents and allergens. Therefore, its importance in the immune defense system of the body is immense. Mucosa is often the first line of defense against infection and only when the mucosal barrier breaks down infection may occur. Consequently, in the generation of suitable vaccines against pathogens whose main entry route is the mucosal layer, it is very important to assess mucosal immune responses to immunization.

The mucosal defense system is very complex. Immunoglobulin A and M (IgA, IgM) are believed to be major players and their role has been elucidated in various systems. It is known that IgA on mucosal surfaces prevents mucosal attachment of bacteria and viruses thus limiting their entry. In addition, both IgA and IgM are important in the so-called mechanism of 'immune exclusion' where antigens are captured during their intraepithelial transport, neutralized and excreted thereby preventing

mucosal damage.¹ The role of IgG antibodies is less defined and it is thought that they may have a role in protecting the female genital tract and the respiratory tract where they can be excreted by passive paracellular transport to neutralize antigens in the lumen.^{2,3} Novel findings, however, that passive transfer of human antibodies against HIV-1 of IgG1 subtype was able to protect macaques against intravenous and intravaginal challenge with SHIV indicate that IgG antibodies following passive transfer can neutralize the virus^{4,5} and blunt viral infection. Furthermore, administration of a cocktail of IgG1 antibodies against human HIV-1 to pregnant macaques prevented neonatal transmission and protected the mothers from intravenous infection.⁶ Caution however has been advised in interpreting the results since it is not clear if the same effect can be achieved by using more virulent viral strains or whether the virus was only suppressed.⁷

It has been proposed that the site of induction determines mucosal immune responses and that primed lymphocytes preferentially accumulate at sites where they have been generated.⁸ Published data have shown that it is possible to induce strong humoral and cellular immune responses by injecting naked DNA intramuscularly, subcutaneously or by a novel jet delivery. However, even though naked DNA vaccines utilizing these routes of immunization generates systemic immune responses sufficient to confer protection, mucosal immunity is not induced. In fact, intramuscular immunization of BALB/c and C57Bl6 mice with naked plasmid DNA (pRc/Y-hsp60) encoding *Yersinia enterocolitica* 60-kDa heat

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shock protein (Y- HSP60) conferred protection against dissemination of this infection in the spleen but not at the site of mucosal entry.⁹

In regards to B cells, the proposed model, based on numerous studies looking into B-cell migration patterns and adhesion molecule expression suggests that primed lymphoid cells from nasal-associated lymphoid tissue (NALT), from Eustachian tube-associated lymphoid tissue (TALT) and bronchus-associated tissue (BALT) preferentially home into nasal and salivary glands, pharyngeal and middle ear mucosa, bronchial and mammary glands, but can also home into urogenital tract. For example, intranasal immunization with a DNA vaccine constructed with the CMV promoter conjugated to env gp160 and rev genes in liposomes induced high levels of mucosal IgA antibodies in feces and vaginal fluids that were able to neutralize HIV-1.¹⁰

Primed B cells from Payer's patches (gut-associated lymphoid tissue, GALT) preferentially home into small intestinal mucosa but can also home into the urogenital tract. This was demonstrated in a study where oral immunization with a rotavirus VP6 DNA vaccine encapsulated in polylactide-coglycolide (PLG) microparticles was able to elicit both rotavirus-specific serum antibodies and intestinal immunoglobulin A (IgA). After challenge at 12 weeks postimmunization with homologous rotavirus, fecal rotavirus antigen was significantly reduced compared with controls. Earlier and higher fecal rotavirus-specific IgA responses were noted during the peak period of viral shedding, suggesting that protection was due to specific mucosal immune response.¹¹

B cells induced in appendix and colonic-rectal solitary follicles (also part of GALT) preferentially home into large intestinal mucosa and the urogenital tract.^{12–14} What that means in terms of mucosal vaccines would be that, intrarectal immunization, for example, would induce high levels of secretory IgA in large intestinal mucosa, some in the small intestine and urogenital tract and none in the mucosa of the airways and mammary glands.

It would appear that the same concept applies to B cells originating from vaginal mucosa. Intravaginal immunization with a DNA plasmid encoding the HIV-1 envelope (pcMN160) induced production of Ag-specific intravaginal immunoglobulins that were able to neutralize HIV-1 *in vivo*.¹⁵ In another study, immunization was induced by using gene gun technology via vaginal mucosa, Payers patch or abdominal skin. The results showed that even though immunization via either route induced vaginal antibodies, vaginal antibody titers of IgA and IgG were only consistently sustained if elicited via vaginal route.¹⁶

Even though the same general rule appear to apply to T cells as well, the situation is much more complex. While activated T cells re-enter lymphoid tissues and preferentially accumulate in the lymphoid tissue where the initial activation took place, memory T cells migrate continuously and randomly, similar to naïve T cells.^{17,18} The implication in terms of vaccine development would be that, in the initial phase of an immune response, once primed, Ag-specific T cells would randomly enter and leave various lymphoid compartments but would be preferentially retained in the lymph node where the antigen was presented at first.¹⁹

Inducing mucosal immunity by various DNA vaccine modalities

The discovery that inoculation of naked DNA leads to long-lasting and strong immune responses had generated enthusiasm to assess this vaccine modalities in mice as well as in higher mammalian species (Table 1).

Immunization with naked DNA

DNA can be introduced to the body by a simple intramuscular or intradermal injection or by propelling DNA-coated particles in various tissues,²⁰ most commonly the skin. Professional antigen presenting cells (APCs) are essential for priming immune responses, either through direct transfection of APC,²¹ cross-priming²² or both. The DNA is transcribed and the RNA translated into the host and the degradation of the protein in the proteasome generates peptides that are presented in the context of MHC Class I molecules. Even though the inoculation leads to transfection of a limited number of cells, it still initiates powerful responses. Intradermal and intramuscular gene vaccination of mice induces antigen-specific Th1 cells that secrete high levels of IFN γ ^{23,24} and stimulates production of antibody of the IgG2a isotype^{24,25} while immunization with soluble protein antigens induces predominantly IgG1 and IgE antibodies and Th2 cells that secrete IL-4 and IL-5. It has also been shown that DNA vaccination appears to be highly effective in inducing MHC Class I-restricted, CD8⁺ cytotoxic T lymphocyte (CTL) responses in mice as well as in non-human primates. CTL responses induced by DNA vaccination can last for more than 1 year.²⁶ The effect of DNA vaccination is dominant and it has been shown that pre-immunization with DNA prevents subsequent induction of Th2 responses and induction of antigen specific IgE when a protein in alum is given.²³ While intradermal and intramuscular injection of naked DNA induces predominantly Th1 response, gene immunization of mice with bolistic delivery into the skin with DNA-coated gold microspheres induces a Th2 response.²⁷ It is believed that in this case the small amount of DNA transfected is insufficient to provide the adjuvant effect necessary for induction of Th1 response. Naked DNA is increasingly tested as a vaccine against infectious diseases but it was also used in immunizing against cancer and inducing tolerance to allergens.^{28,29}

ACA mice immunized with plasmid DNA expressing the Epstein-Barr virus (EBV) nuclear Ag-4 (EBNA-4) were partially protected against syngeneic mammary carcinoma line (S6C) expressing EBNA-4 (S6C-E4). This protection was enhanced by co-immunizing mice with EBNA-4 and GM-CSF-expressing plasmids and a full protection was achieved by co-immunizing mice with EBNA-4 and IFN γ -expressing plasmids. Mice that have rejected the EBNA-4-positive tumor were also resistant against a subsequent challenge with the original non-transfected tumor line. This response was long-lived as it was found that even 3 months after the last immunization, full protection was achieved.²⁸

A very interesting study used nanoparticles containing DNA coding for a major peanut allergen (Arah2) complexed with chitosan, a natural polysaccharide derived from crustacean shells to orally feed mice rendered allergic to peanut. Chitosan served as delivery vehicle promoting adhesion to the mucosa and transport through

Table 1 Immune responses to genetically engineered vaccines

<i>Antigen</i>	<i>Vector</i>	<i>Immunization route</i>	<i>Effects</i>
Glutathione S-transferase of <i>Schistosoma haematobium</i>	rBCG	i.n.; i.p.	Neutralizing antibodies of IgG1, IgG2a, IgG2b and IgA in serum and IgA in bronchoalveolar fluids
SIVmac251env, gag, nef	rBCG	oral	CTL responses in Payer's patches, intestinal mucosa
<i>Escherichia Coli</i> β galactosidase <i>Escherichia Coli</i> β galactosidase and <i>Listeria</i> <i>Monocytogenes actA</i> and <i>hly</i> genes	<i>Shigella Flexneri</i> <i>Salmonella</i> <i>Typhimurium</i>	oral	Strong cellular and humoral response Strong CTL and proliferative responses in spleen and mesenteric lymph nodes and specific antibodies in sera
Fragment C of tetanus toxin	<i>Salmonella</i> <i>Typhimurium</i>		
<i>Onchocerca Volvulus</i> glutathione S transferase	<i>Salmonella</i> <i>Typhimurium</i>	oral	Strong proliferative responses in PBMCs and spleen; specific antibodies in sera
Rotavirus VP6 DNA encapsulated in polylactide-coglycolide microparticles		oral	Rotavirus specific serum antibodies and intestinal IgA, reduction of fecal rotavirus antigen
Plasmid DNA encoding <i>Yersinia Enterocolitica</i> heat shock protein (Y-HSP60)		i.m.	Protection against disseminated infection in spleen but not at mucosal sites
Plasmid DNA encoding env GP160 and rev of HIV-1 virus in liposomes		i.n.	High levels of mucosal IgA antibodies in feces and vaginal fluids able to neutralise HIV
Plasmid DNA encoding human growth hormone under the control of cytomegalovirus promoter		Intravaginal, intradermal (abdominal skin), Payer's patches	Ag-specific IgA and IgG antibody titers consistently sustained only via vaginal route
Env and gag genes of HIV-1	Naked DNA priming, boosting with rFowl poxvirus	i.m.; gene gun into abdominal epidermis	Protection from i.v. challenge with rFPV expressing HIV-1 antigen and i.v. challenge with HIV-1
SIV gag-derived multicytotoxic T-lymphocyte epitope	Priming with DNA, boosting with rMVA		Induction of CTL's, no protection after intrarectal challenge
SIV protein, p17 (gag) and gp41 (env)	Recombinant poliovirus	i.n.; i.v.	Strong IgA antibodies in rectal and vaginal secretions, IgG antibodies in sera, anti-SIV T cell proliferative responses
DNA encoding for peanut allergen (Arah2) complexed with chitosan		oral	Allergic reaction to peanut Arah2 protein significantly diminished, increased IgA, IgG2a-Th1 type response
Melanocyte differentiation antigens: GP100, MART-1, tyrosinase and tyrosinase-related proteins (TRP-1 and TRP-2)	Recombinant vaccinia virus	i.v.	Full protection from s.c. challenge with B16 tumor cells, partial protection if injected i.v., vitiligo in mice immunized with rVV encoding in mTRP-1
Human myelin basic protein (hMBP) or hMBP as a fusion protein with β -glucuronidase	<i>Lactobacillus casei</i> with parenteral surface-anchored tetanus toxin fragment C		Secretion of human myelin basic protein (can be used to induce peripheral T cell tolerance)
EBNA-4 gene of Epstein-Barr virus		i.m.; gene gun	Partial or full protection against the outgrowth of S6C-E4, a nonimmunogenic tumor transfected with EBNA-4
Melan-A (membrane protein expressed in melanosomes and endoplasmatic reticulum)	Recombinant vaccinia virus	intraperitoneal	Melan-A-specific tumor-reactive CTL's efficiently induced upon stimulation of PBMC from melanoma patients. High levels of peptide-specific lysis for splenic T cells
SIV gag, pol, env genes	Recombinant poxvirus (NYVAC)	i.m.; CTL and PCR	Protection against i.r. challenge. Increased survival following i.v. challenge
HIV-1 gag, pol, env genes	Recombinant canarypox	i.m.	Cross-protection against i.v. challenge with HIV-2
Rabies virus glycoprotein of the ERA strain	Replication-defective adenoviral recombinant	i.n., s.c., vaginal and intrarectal	s.c. and i.n. induced serum and mucosal antibodies and i.n. immunization induced higher titres of vaginal and intestinal antibodies of IgA type
Plasmid DNA encoding HIV-1 envelope (pcMN160)		intravaginal	Ag-specific intravaginal antibodies able to neutralise HIV <i>in vivo</i>

the epithelium. In control animals, challenge with Arah2 caused severe anaphylactic reaction with increased plasma histamine concentration, increased vascular permeability and raised peanut-specific serum IgE. This reaction was substantially diminished in animals pre-treated with chitosan-DNA particles. It was shown that the immunization may be improving local defense mechanisms by increasing production of fecal IgA and serum IgG2a, decreasing IgE and directing the response to Th1. This study describes a potentially new way to use DNA immunization in inducing tolerance to antigens and may potentially be very useful in oral tolerance to respiratory allergens. The use of chitosan enables oral feeding of the DNA and efficient DNA transfection of epithelial cells.²⁹

While the application of DNA vaccines by intramuscular or intradermal inoculation targets a relatively limited amount of antigen presenting cells, delivery of DNA vaccines by carriers could increase the number of transfected APCs in immunologically relevant organs such as the gut and the gut-associated lymphoid tissue increasing the immune response. These strategies may help the development of vaccines that could be administered orally. Both bacteria and viruses have been used as vector carriers to deliver DNA.

Viruses as delivery vectors

Viruses adhere and penetrate the cell membrane through viral receptors. Recombinant viruses in which foreign DNA is inserted function as vehicle to transport the foreign DNA into the cell. The foreign DNA is expressed by using the cellular machinery and the protein transported through the endogenous pathway of the APCs where it combines with the MHC Class I molecules and is presented to CD8⁺ T cells.³⁰ Various viral carriers have been successfully used and have shown capacity to elicit strong immune responses. Members of the poxvirus family such as attenuated vaccinia derivative and avipox viruses are the most widely used. Vaccinia recombinant and its derivative have a broad host range and have been used for the development of human and animal vaccines. Recombinant vaccinia virus is used as an effective oral wildlife rabies vaccine³¹ and is currently investigated as an HIV vaccine.

Consecutive immunization involving priming with DNA and boosting with recombinant Fowl pox virus (rFPV) vaccines encoding common HIV-1 antigens induced greater HIV-1-specific immunity in mice than both vectors alone and protected them from challenge with a recombinant vaccinia virus expressing HIV-1 antigens. This vaccine regimen also protected macaques from an intravenous HIV-1 challenge.³² High levels of CTLs specific for a single simian immunodeficiency virus (SIV) gag-derived epitope were elicited in rhesus macaques by using a multicytotoxic T- lymphocyte (CTL) epitope gene and vaccination regimen using priming with DNA and boosting with recombinant modified virus Ankara (MVA; an attenuated vaccinia virus). These vaccine-induced CTLs were capable of killing SIV-infected cells *in vitro*. As shown by FACS analysis of tetrameric major histocompatibility complex-peptide complexes, the vaccinated animals had 1 to 5% circulating CD8⁺ lymphocytes specific for the vaccine epitope, frequencies comparable to those in SIV-infected monkeys. However, upon intrarectal challenge with pathogenic SIVmac251, no evidence for protection was observed in at least two of the three

vaccinated animals.³³ A different study described enhanced responses with an optimized vaccine regimen that primes with DNA-Env, boosts with recombinant vaccinia virus-Env, and re-boosts with purified Env proteins.³⁴

Recombinant vaccinia virus is the most widely used vector in design of antitumor vaccines. Immunization against self antigens such as melanocyte differentiation antigens encoded in rVV was shown to induce full protection in mice challenged with tumor cells.³⁵ Unfortunately, this vaccine also induced vitiligo-like symptoms. Recombinant vaccinia virus (rvv-GM-CSF) expressing the granulocyte macrophage stimulating factor (GM-CSF) as tumor vaccine was evaluated in the murine B16-F10 melanoma model. The vaccine was prepared by infection of irradiated tumor cells with rvv-GM-CSF. Immunization of C57BL/6 mice with this construct inhibited tumor development, lung metastasis and prolonged survival. The effect was tumor-cell specific. In another study, strong tumor-specific CTL responses were elicited by using recombinant vaccinia encoding amino acid sequences corresponding to natural CTL-defined peptides from tumor antigens such as melan-A peptide analogue. The use of mutated minigene construct lead to improved antigen recognition and enhanced immunogenicity.³⁶

The avipoxvirus has limited host range and its replication is abortive in mammalian cells. This property has been exploited to generate a safe live attenuated vaccine for humans.³⁷ The most widely tested to date has been canarypox virus-based vaccine that was able to cross-protect not only against HIV-1 but also against HIV-2 viruses in macaque models and is currently undergoing phase 2 trials.^{38–41}

Adenoviruses that replicate in the gut have great potential in eliciting mucosal immune responses and were proven very effective and safe as vectors for viral vaccines. The antigenic diversity (over 45 serotypes described) allows the exploitation of antigenically distinct vectors for multiple sequential booster immunizations. Recombinants using adenovirus as vector have been described and used for a variety of animal viruses including hepatitis B, hepatitis C, vesicular stomatitis, herpes simplex, rabies, parainfluenza and HIV.^{42–44} Adenoviral vectors have also been used to induce antitumor immunity. High efficiency transfection of IL-2 gene into glioblastoma cell lines using adenoviral vectors induced production of high levels of IL-2 and activation of CD8⁺ T cells suggesting generation of antitumor response.⁴⁵ Recently published study where replication-defective adenoviral recombinant was used to induce immune responses against rabies, has shown that it is possible to induce good mucosal immune responses using non-replicative adenoviruses by immunizing intranasally.⁴⁶

Since most HIV-1 infection occurs mucosally, it may be necessary to develop immunization strategies in order to elicit strong mucosal as well as systemic responses and viral recombinants may be a good tool in this endeavour.⁴⁷

This issue was addressed in studies that utilise polioviruses as carriers to immunize against SIV (a virus that induces AIDS-like disease in monkeys⁴⁸) by mucosal route. Intranasal immunization with this construct elicited strong IgA responses in rectal and vaginal secretions and IgG antibodies in sera. Anti-SIV T cell proliferative

responses in PBMCs were also induced.⁴⁹ However, efficacy studies for this vaccine are not yet available.

Herpes simplex virus (HSV) that is capable of a single round of infection (DISC-HSV) was used as a vector to elicit antitumor effect in colorectal cancer. Clear antitumor effects in tumor prevention and tumor therapy were shown with DISC-HSV/GM-CSF.⁵⁰

Plasmid DNA vectors that contained replicons derived from prototype alphavirus, Sindbis and Semliki Forest virus were used to enhance immunogenicity of nucleic acid vaccines. These plasmids, when transfected into cells launch a self-replicating RNA vector (replicon) that, in turn, directs the expression of a model tumor antigen. This strategy elicited immune responses at doses 100 to 1000 fold lower than conventional DNA plasmids.⁵¹

Bacteria as vectors

Mechanisms by which bacteria deliver DNA to host cells is largely dependent on the property of the bacterium itself. Various bacteria have been recently successfully used to deliver DNA. So far, *Mycobacterium Bovis* Bacillus Calmette-Guerin, *Listeria Monocytogenes*, *Salmonella Typhimurium* and *Shigella Flexneri* are the ones in which most of the studies have been done.

Most of the bacteria used as vaccine vectors have been engineered to express the desired protein by using prokaryotic promoters. However, other studies have shown that immune responses can also be elicited by inserting plasmid DNA with an eukaryotic promoter so that the bacterium is only a vehicle for the DNA.

rBCG has been successfully used to induce immune responses to glutathione S-transferase from *Shistosoma haematobium* (Sh28GST), the etiologic agent of human urinary schistosomiasis⁵² and to SIVmac251env, gag and nef proteins.⁵³ After oral ingestion of rBCG SIV nef strain, rBCG entered oropharyngeal and intestinal mucosa, was disseminated to Payer's patches and systemic organs where it multiplied and persisted for a long time inducing CTL responses. Also, large numbers of rBCG remained in the intestinal lumen inducing Ag-specific proliferation of intra-epithelial lymphocytes.⁵⁴ Epitopes from the HIV virus, such as the nef protein⁵⁵ and the Env protein V3 region⁵⁶ have also been used to successfully engineer vaccines using rBCG as a vector.

Attenuated strains of gram-negative bacteria, such as *Salmonella Typhimurium* and *Shigella Flexneri* have also been used to deliver DNA. *S. Flexneri* enters the cell cytosol after phagocytosis and delivers plasmids directly to the intracellular compartment. An invasive phenotype of bacteria is necessary for successful plasmid delivery. Intranasal immunization with *Shigella* induced strong cell and humoral responses against *Escherichia Coli* β -galactosidase.⁵⁷ Similarly, *Shigella Flexneri* attenuated by deletional mutation of *asd* gene harboring a DNA measles virus vaccine plasmid can induce strong virus-specific CD8⁺ CTL responses and IFN- γ secretion in splenocytes.⁵⁸

S. Typhimurium, on the other hand, remains in the phagosomal compartment of the host cell.⁵⁹ Nonetheless, this bacterium still has the ability to deliver eukaryotic expression vectors not only to macrophages but also to dendritic cells.^{60,61} *S. Typhimurium* has been successfully used as a vector to deliver several foreign antigens and to elicit immune responses.

Live *Salmonella Typhimurium*, attenuated by deletional

mutation of the *aroA* gene causing requirement for aromatic metabolites (SL7207) has been used as carrier for the pCMVbeta vector that contains the β -galactosidase (beta-gal) gene,⁶⁰ attenuated *Salmonella Typhimurium* strain CVD 915 was used to deliver plasmids pTETnir15 and pcDNA3tetC encoding fragment C (Frag C) of tetanus toxin under the control of prokaryotic or eukaryotic promoters⁶² and attenuated *Salmonella typhimurium* expressing OvGST was also able to induce cellular and humoral responses in mice to *Onchocerca volvulus* glutathione S-transferase (OvGST).⁶³

Attenuated intracellular bacteria can accomplish delivery of DNA to APCs. Dietrich et al⁶⁴ engineered the intracellular pathogen *Listeria Monocytogenes* to deliver plasmid DNA into the cytosol of macrophages. After inoculation, these bacteria lyse the vacuole of the macrophage and enter the cytosol where they grow. *Listeria* also has the ability to spread from cell to cell without contacting the extracellular milieu. In this model, immunity is mediated by CD8⁺ T cells and antibodies do not play a significant role.

In addition, recombinant *Lactobacillus casei* which expresses surface-anchored tetanus toxin fragment C and secretes human myelin basic protein was generated. It is proposed that such a construct may be useful in inducing peripheral T cell tolerance and protection against autoimmune diseases as well as in oral vaccination against tetanus.⁶⁵

Enhancing and modifying mucosal immune responses

Several approaches have been used to enhance and/or modify mucosal immune responses.

The potency of Th1 vs Th2 cytokines to enhance immune responses to DNA vaccines was examined in a study where DNA expression construct encoding the HSV-2 gD protein (a glycoprotein involved in HSV binding and entry into the host cell) was codelivered with the gene plasmids encoding the Th1-type (IL-2, IL-12, IL-15 and IL-18) and Th2-type (IL-4 and IL-10) cytokines by intramuscular injection. Th1 cytokine gene co-administration not only enhanced the survival rate but also reduced the frequency and severity of herpetic lesions following intravaginal HSV challenge. On the other hand, co-injection with Th2 cytokine genes increased the rate of mortality and morbidity of the challenged mice. IL-12 was a particularly potent adjuvant for the gD DNA vaccination.⁶⁶ In a follow-up study, co-injection with IL-12 cDNA and gD DNA vaccine inhibited both systemic gD-specific Ab and local Ab levels compared with gD plasmid vaccination alone. In contrast, Th1 cell proliferative responses and secretion of cytokines (IL-2 and IFN γ) and chemokines (RANTES and MIP-1 α) were significantly increased by IL-12 coinjection. IL-12 coinjection with a gD DNA vaccine showed significantly better protection from lethal HSV-2 challenge compared with gD DNA vaccination alone in both inbred and outbred mice. This enhanced protection appears to be mediated by CD4⁺ T cells, as determined by *in vivo* CD4⁺ T cell deletion.⁶⁷

In a study in the macaque model, the gag, pol, and env genes of SIV(K6W) were expressed in the NYVAC vector, a genetically engineered derivative of the vaccinia virus Copenhagen strain that displays a highly attenuated

phenotype in humans.⁶⁸ In addition, the human IL-12 p35 and p40 subunits as well as the IL-2 gene, were expressed in separate NYVAC vectors and inoculated intramuscularly, in conjunction with or separate from the NYVAC-SIV vaccine. The overall cytotoxic T-lymphocyte (CTL) response was greater, at the expense of proliferative and humoral responses, in animals immunized with NYVAC-SIV and NYVAC-IL-12 than in animals immunized with the NYVAC-SIV vaccine alone. Significantly, five of the 11 vaccinees exposed mucosally to SIV(mac251) showed a transient peak of viremia 1 week after viral challenge and subsequently appeared to clear viral infection. In contrast, all 12 animals inoculated intravenously became infected, but 5 to 6 months after viral challenge, four animals were able to control viral expression and appeared to progress to disease more slowly than control animals.⁶⁹ Similarly, IL-12 delivery from recombinant vaccinia virus attenuated the vector and enhanced the cellular immune response against HIV-1 Env in a dose-dependent manner.⁷⁰

In another study, mucosal immunization of vaccinia-immune BALB/c mice with recombinant vaccinia expressing HIV gp160 induced specific serum antibody and strong HIV-specific cytotoxic T lymphocyte responses. These responses occurred not only in mucosal but also in systemic lymphoid tissue. The systemic HIV-specific cytotoxic T lymphocyte response was enhanced by coadministration of IL-12 at the mucosal site.⁷¹

When HIV-1 envelope DNA vaccine and interleukin 15 (IL-15) expression plasmid were intranasally administered to mice, a significant increase in the HIV-1-specific DTH response and CTL activity, and decrease in the serum IgG/IgG2a ratio was observed in the group which received DNA vaccine and IL-15 expression plasmid compared to DNA vaccination alone. Restimulated immune lymphoid cells from mice that received both agents showed enhanced production of interferon-gamma (IFN-gamma) and reduced secretion of IL-4.⁷²

The importance of secretory IgA at mucosal surfaces was discussed earlier. A study describing an increase in specific local IgA responses to heterologous antigen in the lungs when genes for murine IL-5 and IL-6 in recombinant vaccinia virus were co-expressed and mice were inoculated intranasally, points out that an opportunity may exist to specifically increase the desired immunoglobulins in the mucosa.⁷³ In another attempt to increase intestinal immune responses BALB/c mice were immunized with HIV-DNA vaccine formulated with a polymer, low-viscosity carboxymethylcellulose sodium salt (CMCS-L) via the intranasal (in.) route. The combination with the polymer elicited higher levels of antigen-specific serum IgG and fecal IgA antibodies than DNA vaccine alone and HIV-specific delayed-type hypersensitivity response and cytotoxic T lymphocyte activity were enhanced.⁷⁴ Mucosal secretory IgA can also be enhanced by co-inoculating MIP-1 α expression plasmid with the DNA vaccine.⁷⁵

As genetic engineering evolves, it opens exciting possibilities to create a completely safe and effective vaccine, to boost specific immune responses at desired sites, and/or to enhance the desired effects, thus limiting the unwanted reaction of the mucosal tissue.

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