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Vaccine 20 (2002) 2007–2010

Vaccine

www.elsevier.com/locate/vaccine

Note

Engineered *Listeria monocytogenes* as an AIDS vaccine

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Received 7 June 2001; accepted 28 November 2001

Abstract

Listeria monocytogenes (Lm) is an attractive vector to elicit T cell immunity because it infects antigen-presenting cells and because infection originates at the mucosa. Lm expressing HIV gag elicits sustained high levels of gag-specific CTL in mice. Since Lm causes disease in immunocompromised hosts, a highly attenuated strain of Lm that requires D-Ala for viability was produced. Attenuated bacteria expressing HIV-1 gag (*Lmdd-gag*) are as efficient as wild-type recombinants at stimulating gag-specific murine CTL when administered with D-Ala and at boosting human CTL in vitro. *Lmdd-gag* immunization protects mice from vaccinia-gag challenge and induces mucosal CTL, even after systemic immunization.

Keywords: *Listeria monocytogenes*; D-Ala; *Lmdd-gag*; HIV; Oral vaccine; Mucosal immunity

1. Rationale for a T cell vaccine for HIV

In the past, most vaccine development efforts have focused on inducing humoral immunity as a critical component of protective immunity. For many viral infections, however, viral-specific T cells provide protection. This has been demonstrated most cogently in murine models of influenza A, LCMV, CMV, RSV and Sendai virus in which adoptive transfer of viral-specific CD8 T cells or immunization to elicit a response to a viral CD8 T cell epitope provides protection against lethal challenge [1–6]. A recent study also showed protection against infection with the bovine leukemia virus in sheep immunized with a CD8 T cell epitope vaccine [7]. Although live attenuated vaccines in clinical use for protection against viral (measles, mumps, rubella, rabies, oral polio and vaccinia) and intracellular bacterial (BCG, oral typhoid) infections would be expected to elicit a strong T cell response, these vaccines were developed by measuring antibody titers and showing protection from challenge. In fact, little is known about the specific T cell response to these vaccines.

The first decade of HIV vaccine development focused on generating humoral immunity with protein subunit vaccines to the viral envelope. These approaches have been foiled by the genetic variability of HIV envelope and by difficulty generating antibodies capable of neutralizing strains of the virus that are not adapted to laboratory growth. Frustration with this approach, together with increasing evidence for an important role for T cells in protection from infection in exposed but uninfected individuals and in the control of the initial viremia of primary HIV and SIV infection, has directed efforts towards developing HIV vaccines capable of eliciting a strong T cell response. Although subunit vaccines can prime antigen presenting cells (APCs) for presentation and activation of CD4 T cell responses, they do not efficiently induce CD8 T cell responses. CD8 T cell priming requires antigen delivery to the cytosol for processing into the MHC class I pathway. Attenuated live vectors are the most potent inducers of CD8 T cells, but introduce safety concerns.

An ideal live vector for an HIV vaccine designed primarily to induce T cell immunity would have the following properties: preferential infection of professional APCs, mucosal infection, low incidence of natural infection in the target population (to reduce prior immunity to the vector itself), well-developed strategies to insert and express foreign genes efficiently in the cytosol of infected cells, safety when administered to humans of varied immune status, stability under varied storage conditions, and low production cost.

2. Advantages of Lm as an HIV vaccine vector

Lm scores highly on this checklist. Lm is an attractive candidate vaccine vector for inducing strong CTL, CD4 helper

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0264-410X/02/$ – see front matter
PII: S0264-410X(02)00088-9

and possibly antibody responses. Inoculation with recombinant bacteria that express foreign proteins provides protection to mice challenged with tumors, LCMV, vaccinia and influenza [8–12]. The natural route of infection is oral and therefore, the induction of mucosal immunity should be optimal. It specifically infects professional APCs, monocytes, macrophages and dendritic cells [13]. After it is taken up by monocytes/macrophages some of the organisms escape the phagocytic vacuole, grow in the cytosol and present secreted protein antigens to the class I pathway to CD8+ CTLs. Lm has a well-defined secretory pathway for expressing genes in the cytosol of the host cell for antigen processing into the MHC class I pathway [14]. Because Lm is a strong adjuvant, the resultant CTL response is strong and long-lasting. In fact, Lm vectors expressing gag elicit a powerful and sustained HIV-specific CTL response in mice [15–17] and boost the human gag-specific CTL response in vitro. At the same time, most of the bacteria remain in the vacuole where they present the same secreted proteins as class II antigens to CD4+ T helper cells. The CD4 T cell response is directed towards a T_H1 response, the response best suited for antiviral protection. Moreover, the incidence of listeriosis is low [18] and therefore preexisting immunity to the vector should not be widespread (although this has not been formally studied).

From a pragmatic point of view, the bacterium is easy to engineer. A shuttle vector methodology has been developed to easily insert foreign genes stably into the bacterial chromosome with high levels of protein expression [15,19]. Lm can also be used as a carrier of plasmid DNA encoding foreign antigens [20]. Bacterial vectors are inexpensive to manufacture and suitable for large scale use in developed and developing countries. Unlike viral vectors, bacterial vectors, including Lm, have the safety feature of being readily treated with antibiotics. Moreover, as a gram + bacterium, Lm is endotoxin free.

3. Attenuation of Lm for vaccine use

Although Lm does not cause clinical symptoms in most hosts, for use as a live vaccine in humans, this vector must be shown to be not only effective, but safe. Although the incidence of listeriosis is rare (<1 in 100,000 cases per year) [18], infection in immunocompromised hosts, pregnant women and neonates can cause serious infections including meningitis and death. In developing Lm as a vaccine vector Frankel focused on attenuating the bacteria and verifying that attenuation is not at the expense of immunogenicity. In the first generation constructs carrying HIV gag or nef, the expression of foreign genes inserted into the non-essential sepA region of the bacterial chromosome, results in three logs of attenuation, and induces potent T cell responses to the HIV gene products [15]. Other early attempts at attenuation have either been incomplete or have come at the price of reduced immunogenicity. Heat killed bacteria induce protective immunity, but it is short-lived [21]. Genetically modified bacteria with an inactivated *hly* gene are avirulent, but fail to present antigens for CD8 T cell activation [22,23]. Although disruption of *actA* does not interfere with the ability of bacteria to replicate in the cytoplasm and present antigens via the class I pathway, the attenuation may not be sufficient. The LD_{50} of the *actA* mutant bacteria is three logs higher than wild-type bacteria and comparable to that of *Lm-gag* bacteria. However, these bacteria are still virulent and persist in mice in the liver for up to 7 days [24,25].

Because further attenuation was thought necessary, Frankel developed a highly attenuated Lm vaccine vector (*Lmdd*) by knocking out the two genes for D-Ala biosynthesis, required for production of a competent cell wall [26]. The genetically attenuated strain *Lmdd* requires the unusual amino acid, D-Ala, not synthesized by vertebrates, for viability. In the absence of D-Ala, it also cannot escape the host cell vacuole. Moreover, *Lmdd* is unlikely to revert to virulence, since large fractions of the D-amino acid transferase and D-Ala racemase genes are deleted from the bacterial chromosome and these genes are not encoded on bacterial plasmids.

4. Safety and immunogenicity of *Lmdd-gag*

Lmdd-gag does not cause any toxicity in mice at the highest doses tested (>8×10^8 bacteria/mouse i.p., 5×10^9 i.m. or 5×10^{10} i.g., compared to an LD_{50} of 3×10^4 i.p. for wild-type bacteria). Even in the presence of D-Ala, the LD_{50} is 7×10^7 i.p. In neonatal mice (2–5 days of age; body weight, ~1 g), 10 wild-type Lm or 10^2 *Lm-gag* administered i.p. causes death of half the animals by day 4 following infection, while 10^4 and 10^6 of *Lmdd-gag* had no detrimental effect, even in the presence of sufficient D-Ala to induce a strong immune response. However, 10^8 of any of the strains given i.p. to neonatal mice are fatal. Therefore *Lmdd-gag* is attenuated at least five logs even in neonatal mice.

In feeding trials in macaques, doses of 10^5 or 10^7 of wild-type Lm produced no detectable effects, although 10^9 organisms caused irritability, loss of appetite and occasional diarrhea [27]. Some infections of macaques (Paterson, Frankel, and Murphey-Corb, unpublished results) were performed using recombinants of wild-type Lm that expressed SIV gag or SIV env. These bacteria have an LD_{50} that is about (100–1000)× higher than that of non-recombinant Lm, presumably because of the deleterious effect of the forced expression of foreign genes. Monkeys were primed orally with 10^9 or 10^{10} bacteria and boosted with 10^{10} or 10^{11} bacteria, respectively with no observable effect on the health of the animals. The same numbers of organisms were also administered i.v. to another group of animals, again with no detectable health effect. Safety and immunogenicity studies of *Lmdd-gag* in rhesus macaques are about to begin.

Immunization of mice with recombinant *Lmdd* bacteria encoding HIV gag (*Lmdd-gag)* elicits incredibly strong and prolonged CD8 T cell responses both systemically and in

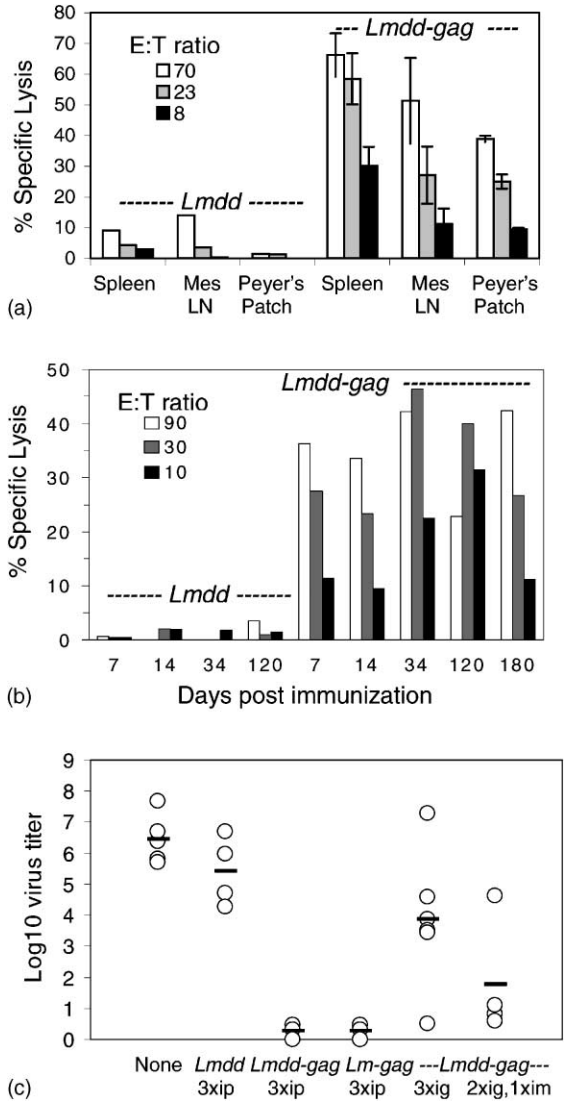

Fig. 1. *Lmdd-gag* induces a strong, durable and protective CTL response in mice: (a) intragastric inoculation of BALB/c mice twice with *Lmdd-gag*, but not with the vector *Lmdd*, induces a strong systemic and mucosal gag-specific CTL response. Lymphocytes from spleen, mesenteric LN and Peyer's patches were cultured in vitro for 3–6 days with the immunodominant gag peptide and assayed for peptide-specific lysis by 4 h ^{51}Cr release assay; (b) a single i.p. inoculation of *Lmdd-gag* with D-Ala induces long-lived gag-specific CTLs in the spleen; (c) mice immunized with *Lm-gag* or *Lmdd-gag* via different routes were challenged i.p. with vaccinia virus expressing gag. Viral titers in the ovaries are shown. Only systemic immunization provides systemic protection, but oral immunization protects against intrarectal challenge (not shown). Data is from [17].

Peyer's patches and mesenteric lymph nodes [17] (Fig. 1). Immunogenicity in mice requires a small amount of the D-amino acid to be present in the inoculum to start the infection, which does not jeopardize safety. The induced CTL response is as strong and durable as that induced by viral vectors and significantly more than that induced by other T cell vaccine strategies (DNA vaccination, recombinant anthrax toxin). A single i.p. injection of *Lmdd-gag* stimulates a strong CTL response to gag in BALB/c mice that persists for at least 6 months. Comparable levels of CTL activity are detected in the spleen, mesenteric LN and Peyer's patches after two or more i.p. injections. The levels of specific CTL are as high as those induced with *Lm-gag*, provided D-Ala is provided in the inoculum. Moreover, oral immunization results in powerful CTL responses to gag in Peyer's patches and mesenteric lymph nodes. However, after oral immunization alone, high levels of mucosal CTL activity are detectable only for the first week after immunization. Nonetheless, these animals are protected from oral challenge 43 days later [17]. The hyper-attenuated strain is as efficient at CTL induction as the unattenuated strain. We have also found similar results boosting human HIV-specific CTLs in vitro [28]. Initial studies have focused mostly on CTL induction; further studies are needed to look at the induction of viral suppressor activity, cytokine and chemokine secretion by both CD4 and CD8 T cells. Although most studies have been done with bacteria expressing gag, the first generation nef-expressing bacteria also efficiently boosts nef responses in human CD8 T cells in vitro.

Immunization with *Lmdd-gag* protects mice from challenge with vaccinia expressing gag [17] (Fig. 1c). Mice immunized with *Lm-gag* or *Lmdd-gag* by various routes were challenged by either systemic (i.p.) or mucosal (i.r.) infection with gag-expressing vaccinia virus. Systemic immunization completely protects mice from either systemic or mucosal challenge. Mucosal immunization provides complete protection from mucosal challenge, but only partial protection from systemic challenge. Neonatal mice are also protected from challenge (data not shown).

5. Summary and future studies

A highly attenuated Lm vector expressing HIV gag was produced by genetically disrupting genes required for the biosynthesis of the bacterial cell wall. The engineered bacterium is attenuated by at least five logs in neonatal and adult mice and induces strong, durable and protective immunity in a heterologous viral mucosal or systemic challenge model in mice. Mucosal T cell immunity is induced even after i.p. inoculation. Macaque safety and immunogenicity studies are about to begin. If *Lmdd-gag* proves safe and immunogenic in macaques, we plan to test the safety and immunogenicity of oral and i.m. administration in a Phase I human study.

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