Attenuated *Salmonella enteritidis* E23 as a vehicle for the rectal delivery of DNA vaccine coding for HIV-1 polyepitope CTL immunogen

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Summary

This study is focusing on elucidation of the capacity of attenuated *Salmonella enteritidis* E23 (*cya, crp*) to serve as a vehicle for the rectal delivery of the DNA vaccine. Earlier for creation HIV-1 candidate DNA vaccine we have designed the polyepitope protein TCI (T-cell immunogen), which comprises over 80 CTL epitopes from subtype A, B and C HIV-1 proteins. The gene coding for TCI protein was used to construct the eukaryotic expression plasmid pcDNA-TCI. The attenuated *S. enteritidis* E23 was transformed by electroporation with recombinant plasmid pcDNA-TCI and the expression of the TCI gene was determined *in vitro* and *in vivo*. BALB/c mice were rectally immunized with *S. enteritidis* E23/pcDNA-TCI (10^8 cfu) twice at 4 week interval. Bacteria were not pathogenic for mice and spontaneously eliminated from mice spleen and liver to 60 days post the immunization. Detectable antibodies were generated in 2 weeks after immunization and their level increased after second immunization. The results of INF-γ ELISpot show that mice immunized with *S. enteritidis* E23/pcDNA-TCI elicited HIV-specific cellular immune response. This study demonstrates that attenuated *S. enteritidis* E23 is an effective live vector for rectal delivery of the DNA vaccine pcDNA-TCI to generate humoral and T-cellular responses against HIV-1.

Introduction

DNA vaccines are regarded as a very promising new direction in designing prevention and therapeutic vaccines against various pathogens (Lu *et al.*, 2008; Liu, 2011). The principle of DNA vaccination is often used in designing of the vaccines against HIV (Girard *et al.*, 2006; Watkins, 2010). Several candidate anti-HIV DNA vaccines have been developed, and the ability of the vaccines to induce an HIV-specific immune response in both laboratory animal models and clinical trials has been demonstrated (Ourmanov *et al.*, 2000; Rerks-Ngarm *et al.*, 2009). In the most of studies the genes coding the HIV-1 full-length proteins, such as Env, Pol, Nef and Vif, have been used for constructing of DNA vaccines. However, construction of artificial polyepitope immunogens might be more promising when designing the vaccines against actively mutating HIV-1. The polyepitope vaccines have certain advantages over the vaccines comprising full-length virus proteins, since they do not contain the regions capable of causing an immune pathology and unite in one molecule the protein fragments of various virus subtypes. Recently, several candidate polyepitope vaccines against HIV-1 containing also the cytotoxic T-lymphocyte (CTL) epitopes have been constructed (Gorse *et al.*, 2008; Spearman *et al.*, 2009; Rosa *et al.*, 2011).

The experiments with DNA vaccination detected certain problems. One of these problems is associated with a low immunogenicity of DNA vaccines; correspondingly, a sufficiently high immune response in the case of intramuscular administration requires injection of rather high doses of plasmid DNA. This suggests the need for the search for alternative delivery routes for the DNA vaccines able to provide a high specific immune response at a small dose of administered DNA. A number of approaches are developed to solve the problem of DNA vaccine delivery, such as electroporation and use of viral or bacterial carriers (Belyakov *et al.*, 2008; Bråve *et al.*, 2010; Pan *et al.*, 2010).

Several authors have demonstrated that orally administered attenuated *Salmonella* strains are one of the promising methods for delivering DNA vaccines (Darji *et al.*, 1997; Loessner and Weiss, 2004; Li *et al.*, 2006; Qu *et al.*, 2008; Ning *et al.*, 2009). These bacteria have widely been used as antigen carriers, they can be easily handled in the laboratory and several well-characterized attenuated strains are available (Curtiss *et al.*, 2010).

In our study we used *Salmonella enteritidis* E-23 (*cya, crp*) developed in Moscow Medical Academy, Russia (Boĭchenko *et al.*, 1995). Strain was attenuated by deletion mutations in genes for adenylate cyclase (*cya*) and the cAMP receptor protein (*crp*). The pathogenicity of *S. enteritidis* E23 is decreased more than 10^7-fold as compared with the parental strain (Boĭchenko *et al.*, 1995).

Live, attenuated *Salmonella* vaccine is also known to induce strong and sustained humoral as well as cellular immune responses both in the mucosal and in the systemic compartments (Shata *et al.*, 2002; Guzman *et al.*, 2006). Furthermore, bacteria are controllable by common antibiotics thus adding additional safety features to these carriers. It should be possible to produce bacterial DNA carriers at low costs and to apply such vaccines orally, this allows an injection administration to be avoided, which is of special importance in the case of mass immunizations covering the risk groups.

All authors of above mentioned studies used *Salmonella* as a carrier for oral genetic vaccination. We chose rectal route of vaccination with *Salmonella* that carry HIV-1 DNA vaccine because intestinal mucosa serve as site for virus entry and are the initial and predominant sites where the virus replicates. Moreover, rectal using of such vaccine requires no antacids for neutralizing an acid gastric pH, as would be necessary in the case of an oral administration.

We have previously described the recombinant plasmid pcDNA-TCI (HIV-1 DNA vaccine) containing artificial gene encoding the polyepitope protein TCI, which comprises over 80 CTL epitopes from subtype A, B and C HIV-1 proteins (Bazhan *et al.*, 2004). In this work, we have studied the immunogenicity of the attenuated strain *S. enteritidis* E-23 as a carrier for rectal genetic vaccination with pcDNA-TCI plasmid encoding the HIV-1 polyepitope CTL immunogen TCI.

Results

Transient expression of pcDNA-TCI in 293T cells and in vitro *assessment of pcDNA-TCI stability*

A synthetic polyepitope T-cell immunogen (TCI) was designed as a candidate DNA-based vaccine against HIV-1 with the emphasis on stimulating CTLs, which play an important role in preventing HIV infection and/or slowing the progression to AIDS. TCI includes fragments from the main virus proteins Env, Gag, Pol and Nef, which contains the epitopes inducing both CD8+ CTL and CD4+ Th (Bazhan *et al.*, 2004). All included epitopes are highly conserved in the three main HIV-1 subtypes A, B and C. To be able to detect CTL responses induced by a DNA vaccine in experimental animals, additional epitopes, restricted by mouse and *Macaque rhesus* MHC class I molecules,

were included in the target immunogen. Resulting artificial protein (392 amino acids in length) contains over 80 CTL epitopes, many of which are overlapping and are totally restricted by 10 different HLA class I (HLA-A, B, Cw) molecules (Fig. 1) (Bazhan *et al.*, 2004).

The gene encoding the polyepitope TCI immunogen was inserted into the pcDNA3.1 vector plasmid, thus giving the DNA vaccine pcDNA-TCI (Bazhan *et al.*, 2004). The expression of TCI gene was demonstrated by immunohistochemical staining. The cytoplasm staining was observed in 293T cells transfected with pcDNA-TCI (Fig. 2A), but not with the parental vector pcDNA3.1 (Fig. 2B).

Assessment of the stability of the plasmid pcDNA-TCI within *S. enteritidis* E23 cells shows that the plasmid persists for at least 100 generations following the exposure to agar media both with and without ampicillin. The authenticity of the plasmid was confirmed by restriction analysis and sequencing.

In vivo *transcription of TCI gene using attenuated* S. enteritidis *E23/pcDNA-TCI as a transgenic vehicle*

Peyer's patches are the main colonization site of the attenuated *S. enteritidis* E23/pcDNA-TCI and an important immunologically relevant site in the context of the mucosal responsiveness. To test an *in vivo* delivery of pcDNA-TCI using attenuated *Salmonella* as a transgenic vehicle, total cellular RNA was isolated from mouse small intestinal Peyer's patches on day 3 after the immunization with attenuated *S. enteritidis* E23/pcDNA-TCI and the TCI gene transcription was analysed by RT-PCR. As it is shown in Fig. 3, only a DNA fragment with a length of about 1191 bp was amplified from RNA of the mice immunized with *S. enteritidis* E23/pcDNA-TCI. Meanwhile, there were no DNA fragments amplified from the RNA prior to reverse transcription with the same primers or from the RNA of the control mice immunized with *S. enteritidis* E23/pcDNA3.1. Murine β-actin DNA fragment (330 bp) was amplified from all samples.

Persistence of S. enteritidis *E23/pcDNA-TCI in mouse body*

BALB/c mice were single or twice rectally immunized with a *S. enteritidis* E23/pcDNA3.1. The colonization and persistence of bacteria *in vivo* was investigated. The performed experiments have demonstrated that *S. enteritidis* E23/pcDNA-TCI was detectable in Peyer's patches from day 2 to day 15. Stating from day 15, the bacteria were detectable in spleen and liver for 42 days in the case of a single immunization and for 63 days in the case of a double immunization. Colonies isolated on differential agar media. It was shown that recovered bacteria colonies
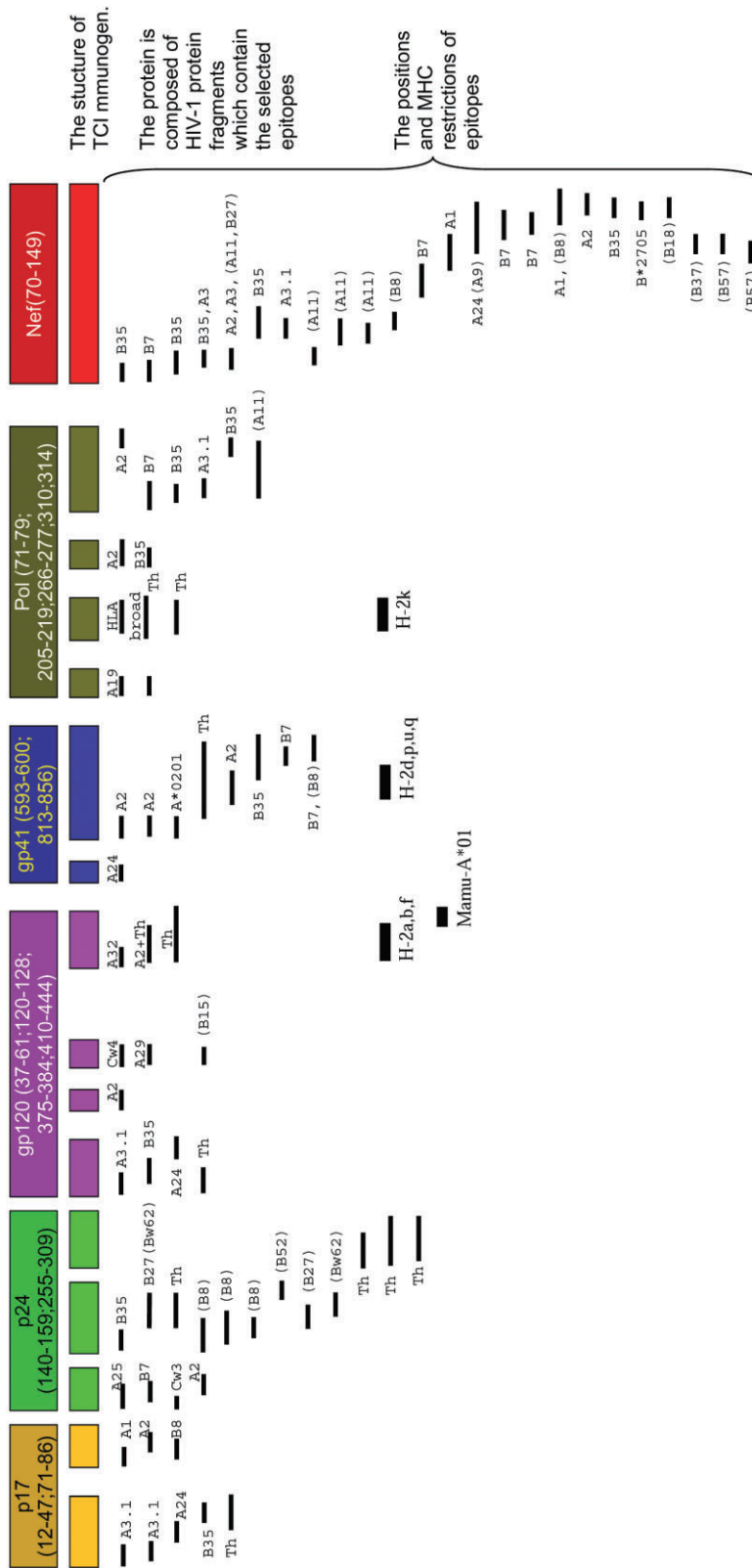
Fig. 1. Design of the TCI immunogen, a candidate for use as an anti-HIV-1 vaccine: a general schematic. Bar patterns indicate the polyepitope CTL immunogen and the origin of the sequences. The positions of individual epitopes and their MHC restrictions (HLA-A, B, Cw – human; H-2a, b, d, f, k, p, u, q – mouse; Mamu-A*01 – *Macaca mulatta*) are depicted as lines below the CTL immunogen. Th stands for helper epitopes. All included epitopes are highly conserved in the three main HIV-1 subtypes A, B and C.
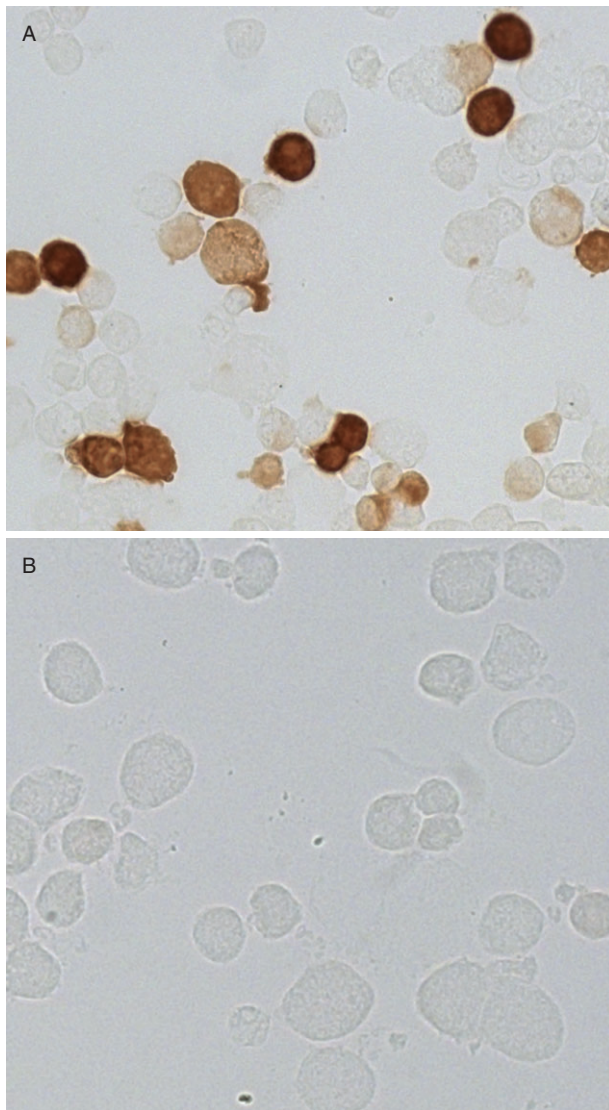
Fig. 2. Expression of TCI gene in 293T cells. Transfected cells were incubated with Mabs against the HIV-1 p24 protein and HRP-conjugated rabbit anti-mouse IgG; complex was visualized by staining with a substrate solution containing 3.3′-diaminobenzidine tetrahydrochloride: (A) 293T cells transfected with pcDNA-TCI and (B) 293T cells transfected with pcDNA3.1. Cells were visualized by microscopy with an Axioskop 2 (Carl Zeiss).

according to cultural and biochemical tests complied with attenuated strain *S. enteritidis* E23. We showed previously that strain *S. enteritidis* E23 avirulent phenotype persists after multifold passages in mouse body (Ryzhova and Boĭchenko, 1997).

Salmonella enteritidis E23/pcDNA-TCI was undetectable by plating of the lung tissue and blood of the immunized animals.

The plasmid DNAs were isolated from recovered bacteria to perform restriction analysis, which demon-

strated that the plasmids were identical to the pcDNA-TCI collection variant (SRC 'Vector', Russia) (Fig. 4).

Humoral immune responses induced by S. enteritidis E23/pcDNA-TCI

The ability of the recombinant *S. enteritidis* E23 to induce the humoral immune response was determined by measuring the level of IgG antibodies to recombinant TCI protein and inactivated HIV-1 lysate in serum samples (Fig. 5A and B). It has been demonstrated that the immunization of mice with the *S. enteritidis* E23/pcDNA-TCI
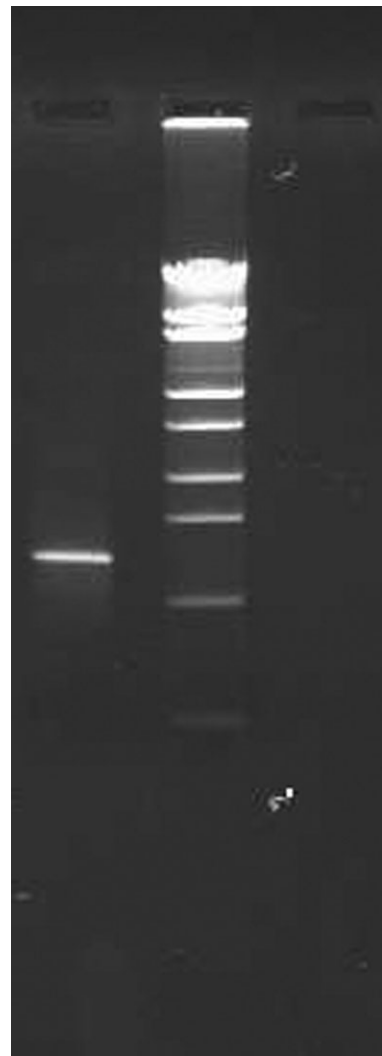

Fig. 3. RT-PCR detection of *in vivo* TCI gene transcription. Electrophoretic pattern of PCR products in 1% agarose gel: lane M, λ/StyI; length of fragments are 19 329, 7743, 6223, 4254, 3472, 2690, 1882, 1489, 925, 421 and 74 bp; lane 1, the PCR product obtained using the RNA from the mice immunized with *S. enteritidis* E23/pcDNA-TCI as a template; and lane 2, the PCR product obtained using the RNA from the mice immunized with *S. enteritidis* E23/pcDNA3.1 as a template.
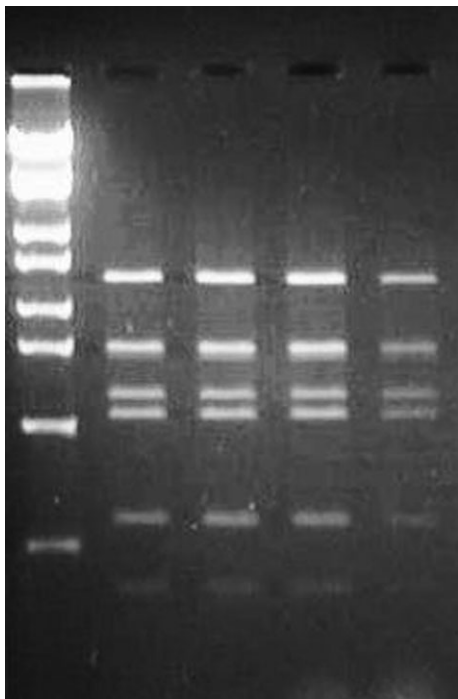
Fig. 4. Electrophoretic pattern (1% agarose gel) of the DNA fragments obtained by hydrolysing the plasmid pcDNA-TCI with the restriction endonucleases BglII and PsiI (lengths of plasmid fragments are 2320, 1468, 1107, 981, 491 and 290 bp): lane 1, plasmid pcDNA-TCI (collection of SRC 'Vector', Russia); lane 2, the plasmid pcDNA-TCI isolated from the liver of the mice immunized with *S. enteritidis* E23/pcDNA-TCI on day 35 after immunization; lane 3, the plasmid pcDNA-TCI isolated from the spleen of the mice immunized with *S. enteritidis* E23/pcDNA-TCI on day 35 after immunization; lane 4, the plasmid pcDNA-TCI isolated from the Peyer's patches of the mice immunized with *S. enteritidis* E23/pcDNA-TCI on day 15 after immunization; and lane M, λ/StyI; length of fragments are 19 329, 7743, 6223, 4254, 3472, 2690, 1882, 1489, 925, 421 and 74 bp.

induces specific antibodies to both the recombinant protein TCI and native HIV-1 proteins. After a single immunization, the specific antibodies at a detectable titre appeared as early as day 16 (Fig. 5A). The maximum antibody titre was observed on day 35 after immunization and amounted to 1:245. The second immunization on day 28 after the first one elevated the synthesis of specific antibodies. The antibody titre reached its maximum values (1:700) on day 35 after the first immunization (day 7 after the second immunization) and then decreased over 28 days to a level of 1:470 (Fig. 5B).

T-cell immune responses induced by S. enteritidis *E23/pcDNA-TCI*

The T-cell immune responses were ascertained by the presence of IFN-γ-producing lymphocytes detected by ELISPOT assay using peptides EPFRDYVDRF from p24, DRVIEVVQGAYRAIR from gp41 and CTEMEKEG-

KISKIGP from RT as HIV-1-specific antigens. In a murine system, these peptides are multiple class I molecules, which can be presented to CTL. Figure 6 demonstrates that immunization with *S. enteritidis* E23/pcDNA-TCI triggers CTL responses in comparison with control groups. These data relate to day 35 (day 7 after the second immunization), when the immune response was the strongest.

Discussion

Most of the AIDS vaccine candidates that are currently in clinical trials around the world are delivered by intramuscular or intradermal injection. This route of administration can produce systemic immunity (antibodies and cell-based immune responses), but does not guarantee a
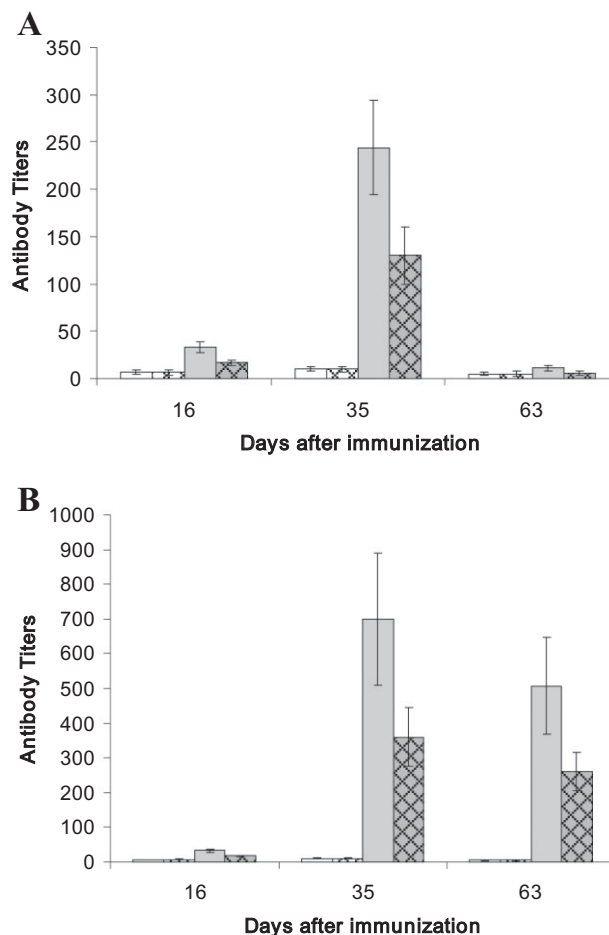

Fig. 5. Total anti-TCI and anti-HIV IgG antibody titres after immunization of mice with the recombinant *Salmonella* (A, single immunization and B, double immunization). Sera were tested for TCI and HIV-1$_{EVK}$ lysate (SRC Vector, Russia). □, anti-TCI and ⊠, anti-HIV antibody in control group of mice immunized with *S. enteritidis* E23/pcDNA3.1; ■, anti-TCI and ⊠, anti-HIV antibody in group of mice immunized with *S. enteritidis* E23/pcDNA-TCI. The data are expressed as mean antibody titres ± SD ($n = 5$).
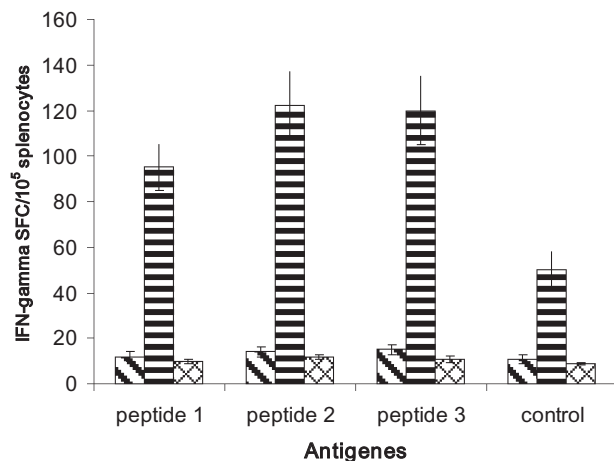
Fig. 6. FN-γ ELISPOT responses 35 days in the BALB/c mice twice immunized with recombinant *Salmonella*. ⊠, mice immunized with *S. enteritidis* E23/pcDNA3.1; ☰, mice immunized with *S. enteritidis* E-23/pcDNA-TCI; ⊠, mice received physiological solution as a negative control. Mouse spleen specimens ($n = 5$) from each group were pooled. For all animals, the splenocytes were separately restimulated *in vitro* with peptide 1 (EPFRDYVDRF), peptide 2 (DRVIEVVQGAYRAIR) and peptide 3 (CTEMEKEGKISKIGP) and EHEC polypeptide as a negative control. The results are expressed as the mean numbers of IFN-γ-secreting cells (spots) per 5×10^5 splenocytes.

robust immune response at the mucosal surfaces. Mucosally administered vaccines could be more effective at producing responses in these tissues.

Recent studies of mucosal vaccine in laboratory animals, including non-human primates, involve vaccination of the gastrointestinal tract and rectum, as well as the nose, oropharynx or respiratory tree in an attempt to elicit responses at the distal mucosal sites where HIV transmission occurs (Duerr, 2010). Currently several scores of different mucosal vaccines are studied such as liposomes, microparticles, different adjuvants, cholera toxin and others (Borges *et al.*, 2010). For instance, Belyakov has demonstrated that intrarectal immunization with synthetic polyepitope peptide AIDS vaccine incorporating the LT(R192G) adjuvant protected rhesus macaques against mucosal infection with simian/human immunodeficiency virus (SHIV) more effectively than the same vaccine given subcutaneously (Belyakov *et al.*, 2001).

The live attenuated bacterial and viral vaccine vectors such as adenoviruses, modified vaccinia virus Ankara, canarypox vectors are currently being developed as AIDS vaccine candidates (Girard *et al.*, 2006).

The live attenuated bacteria display several advantages for vaccine delivery. The vaccines involving live attenuated bacteria are relatively inexpensive for a mass production. Their oral or rectal administration is simple and allows any injections to be avoided. In addition, bacterial possess immunostimulatory molecules, such as lipopolysaccharides (LPS), that can function as adjuvant to stimulate immune responses (Marcela *et al.*, 2003).

A successful use of attenuated *Salmonella* for delivering DNA vaccines has been demonstrated in laboratory animal models for several bacterial, viral and somatic diseases (Darji *et al.*, 1997; Paglia *et al.*, 1998; Flo *et al.*, 2001; Shata *et al.*, 2002; Cazorla *et al.*, 2008; Qu *et al.*, 2008). In all these works, the recombinant *Salmonella* were orally administered. We selected a rectal administration of the *Salmonella* carrying the DNA vaccine against HIV-1, because this is one of the main portal for this virus. In addition, a rectal administration of such vaccine requires no antacids for neutralizing an acid gastric pH, as would be necessary in the case of an oral administration.

The mechanism of DNA vaccine delivery with the help of attenuated bacteria may be as following. After penetrating through the gastric mucosal barrier (mainly via M cells), a large number of the recombinant *Salmonella* is taken up by antigen presenting cells in the local lymphatic tissues, such as Peyer's patches. In these antigen presenting cells, bacteria will start to replicate and die possibly due to their metabolic attenuation. This results in release of DNA vaccine, which provides the production of antigen in antigen presenting cells (Weiss, 2003).

In this work, we used strain *S. enteritidis* E23 (*cya, crp*), obtained from the Sechenov Moscow Medical Academy (Boĭchenko *et al.*, 1995). Strain was attenuated by deletion mutations in genes for adenylate cyclase (*cya*) and the cAMP receptor protein (*crp*). The attenuating mutations Δ*cya* and Δ*crp* are located at a distance of 11 min of genetic map, which excludes the reversion to wild type. These mutations enhance the development of a limited persistence of bacteria in the lymphoid tissue with preservation of their invasive activity. When persisting in macrophages, such mutants have no cytopathic effect on them (Ryzhova and Boĭchenko, 1997). It has been demonstrated in mouse, calf and monkey models that the pathogenicity of this strain is decreased more than 10^7-fold as compared with the parental strain, whereas the invasive activity is preserved. The LD_{50} for *S. enteritidis* E23 intraperitoneally administered to mice amounts to 10^7 colony-forming units (cfu) (Boĭchenko *et al.*, 1995).

Salmonella enteritidis E23 was used as a vector for delivery of the DNA vaccine pcDNA-TCI. Immunohistochemical staining 293T cells transfected with pcDNA-TCI (Fig. 2) and RT-PCR total cellular RNA isolated from Peyer's patches (Fig. 3) have demonstrated expression of the TCI gene. These results convincingly demonstrate the ability of *S. enteritidis* E23 to deliver pcDNA-TCI to the host cells after a rectal immunization.

Note that the DNA vaccine pcDNA-TCI encodes a polyepitope immunogen (TCI) constructed for stimulation of

the HIV-specific CTL response. When constructing the TCI protein, the epitopes from the major HIV-1 (subtypes A, B and C) proteins Env, Gag, Pol and Nef, which induce both the CD8+ CTL and the CD4+ T helpers, were selected (Bazhan *et al.*, 2004; Karpenko *et al.*, 2007). In the sequences of native virus proteins, these epitopes are localized in several continuous regions as partially and sometimes completely overlapping peptides. These continuous regions contain several B-cell epitopes, which overlap with T-cell epitopes. This is why the immunization of mice with the plasmid pcDNA-TCI, coding for TCI protein, is able to induce both the T-cell and the B-cell immune responses against HIV-1.

The next task of our work was to assess the possibility of inducing the immune response in the mice rectally immunized with the recombinant *S. enteritidis* E23 harbouring the DNA vaccine pcDNA-TCI (Figs 5 and 6). It has been shown that a rectal immunization of mice with the help of the *S. enteritidis* E23/pcDNA-TCI induces production of the specific antibodies to HIV-1; moreover, the antibody titres depend on the immunization multiplicity. The second immunization on day 28 after the first one induced an increased synthesis of specific antibodies (Fig. 5B). In addition, the antibody response to the double immunization was more prolonged as compared with a single immunization. The observed decrease in the antibody titre indirectly demonstrates elimination of the DNA vaccine pcDNA-TCI from the body of laboratory animals.

Because interferon-γ is one of the main cytokine, which is secreted by CD8+ T-lymphocytes, the ability of our vaccine construct to induce the CTL response was assessed using ELISPOT by determining the number of lymphocytes producing this cytokine. Peptides EPFRDYVDRF from p24, DRVIEVVQGAYRAIR from gp41 and CTEMEKEGKISKIGP from RT, contained in TCI, were used as specific antigens. It is known that these peptides in mice are restricted by class I and II MHC proteins, which provides the stimulation of both the CD8+ CTL and the CD4+ T helper responses. It is evident from the data shown in Fig. 6 that the response of the splenocytes stimulated by the specific peptides was more higher as compared with the spontaneous (without peptides) control (Fig. 6).

Assessment of the safety of the recombinant *S. enteritidis* E23/pcDNA-TCI has demonstrated that this strain displays a decreased ability to reproduce in the mammalian body. Neither lethal cases nor any other side-effects were detected in the mice that received the bacteria rectally at a dose of 10^8 cfu. The recombinant *S. enteritidis* E23/pcDNA-TCI are eliminated from the body. The bacteria were detectable in spleen and liver for 42 days in the case of a single immunization and for 63 days in the case of a double immunization.

Thus, the study of immunogenic properties of the DNA vaccine encoding HIV-1 polyepitope CTL immunogen within the attenuated strain *S. enteritidis* E23 allows us to make the following conclusions. The recombinant *S. enteritidis* E23 delivers the plasmid pcDNA-TCI into antigen presenting cells and provides expression of the target gene. Immunization of mice with *S. enteritidis* E23/pcDNA-TCI induces production of specific antibodies, which bind to both the recombinant protein TCI and HIV-1 native proteins; and immunization of laboratory animals with *S. enteritidis* E23/pcDNA-TCI induces the specific T-cell response.

Experimental procedures

Plasmids, bacterial strains and media

The eukaryotic expression vector pcDNA3.1 contains cytomegalovirus (CMV) immediate-early promoter for efficient expression and bovine growth hormone (BGH) poly(A) signal for mRNA stability, purchased from Invitrogen, USA. The plasmid pcDNA-TCI was constructed on the base of pcDNA3.1 as described earlier (Bazhan *et al.*, 2004). The attenuated *S. enteritidis* E23 (Δ*cya*, Δ*crp*) was kindly provided by Professor M.N. Boĭchenko, Moscow Medical Academy, Russia (Boĭchenko *et al.*, 1995). Media were prepared as described Sambrook and Russel (2001). The purified plasmid pcDNA-TCI or control vector pcDNA3.1 was transformed into *S. enteritidis* E23 competent cells by electroporation. The positive transformants were selected on LB agar containing 100 μg ml⁻¹ ampicillin and verified by plasmid isolation and digestion with restriction enzymes. *S. enteritidis* E23/pcDNA-TCI was cultivated as earlier described (Ryzhova and Boĭchenko, 1997). For immunization experiments, an overnight bacterial culture was diluted (1:100) with LB broth (Difco) under aeration at 37°C until it reached the mid-log phase, 10^9 cfu per ml. The bacterial cells were collected by centrifugation at 5000 *g* for 10 min and suspended in phosphate-buffered saline (PBS) to assess the cell populations by plating serial dilutions on LB agar.

Transient expression of the recombinant plasmid

The 293T cells were grown in six-well tissue culture plates (Costar) with Iscove's modified Dulbecco's medium (Sigma) supplemented with 10% fetal bovine serum (HyClone) and 50 μg ml⁻¹ gentamicin. Monolayer of 70–80% confluent cells was transiently transfected with the plasmid pcDNA-TCI or empty plasmid pcDNA3.1 using FuGENE® HD Transfection Reagent (Roshe). At 48 h post transfection, the cells were washed with PBS, transferred onto microscopic slides and fixed with ice-cold methanol/acetone (1:1) at 4°C for 30 min. Endogenous peroxidase activity was blocked by incubation in PBS containing 1% H_2O_2 and 0.1% NaN_3. The preparations of transfected cells were pre-incubated for 30 min with 20% FBS and then incubated with MAbs 29F2 against the HIV-1 p24 protein (Vector-Best, Russia) for 1 h at room temperature in PBS containing 2% FCS. The cells were washed three times with PBS and incubated with HRP-conjugated rabbit

anti-mouse IgG (Sigma) for 1 h in PBS containing 2% FCS. The HRP complex was visualized by staining with a substrate solution containing 3.3′-diaminobenzidine tetrahydrochloride (Sigma).

Six-well tissue culture plates (Costar) were seeded with 293T cells. Monolayer of 70–80% confluent cells was transiently transfected with the plasmid pcDNA-TCI or empty plasmid pcDNA3.1 using FuGENE® HD Transfection Reagent (Roshe). The cells were washed with PBS 42 h after transfection, fixed with ice-cold methanol/acetone (1:1) at 4°C for 30 min, and washed with PBS. The TCI gene expression was detected by immunohistochemical staining. MAbs 29F2 to p24 HIV-1 (Vector-Best, Russia) were used as primary antibodies and HRP-conjugated rabbit anti-mice IgG (Sigma), as secondary antibodies.

Mice and immunization protocols

Male BALB/c mice (6 weeks old, 17–22 g), purchased from the animal breeding facility with the State Research Center Vector (Koltsovo, Russia), were kept in animal holding laboratory under controlled conditions and standard mouse diet and water. All the work with animals met the protocols on animal use approved by the IACUC of the State Research Center of Virology and Biotechnology Vector (NIH OLAW registration No. 01A5505-01). Mice were randomly divided into five groups. Two control groups were single or twice rectally immunized with a *S. enteritidis* E23/pcDNA3.1. The second immunization was performed on day 28 after the first one. Two experimental groups were single or twice rectally immunized with *S. enteritidis* E23/pcDNA-TCI. A single dose of *Salmonella* was 30 μl of bacterial suspension, containing 10^8 cfu per mouse. The fifth group of mice received physiological solution as a negative control. On days 0, 16, 35 and 63, five mice from each group were bled from the retroorbital sinus to obtain the blood sera. The spleen was excised on day 35 after immunization to isolate splenocytes for ELISPOT assay.

Studying persistence of strains in laboratory animals

Quantitative accumulation and persistence of *S. enteritidis* E23/pcDNA-TCI were studied in the mouse lungs, liver, Peyer's patches and intestines after both single and double rectal immunizations. The organs were excised under sterile conditions on days 5, 10, 16, 28, 35, 42 and 63; homogenized; and plated 100 μl of homogenized samples onto the enteric differential agar media, bismuth sulfate agar or LB agar containing 100 μg ml^{-1} ampicillin. Bacteria colonies were characterized for compliance with *S. enteritidis* E23 using cultural and biochemical tests according to the protocol, suggested by Boĭchenko and colleagues (1995).

Plasmids were isolated from individual colonies and assayed by restriction hydrolysis with the restriction endonucleases BglII and PsiI to verify the structure of pcDNA-TCI. To detect *in vivo* transcription of the TCI gene, cell RNA was isolated from Peyer's patches obtained from several mice on day 5 after immunization using an RNA Isolation System (Promega, USA). The transcripts of TCI gene were analysed by RT-PCR using the following specific primers, amplifying a fragment with a length of 1191 bp:

5′-CGCGGATCCATGAAAATTCGGTTAAGGCCAGGGG -3′ (forward),
5′-TCCCCCCGGGTCATGGTAACCCTGGTACTAGCTTG TAGCAC-3′ (reverse).

The recombinant TCI protein

The recombinant TCI protein is a GST fusion, its coding sequence was cloned into an IPTG-inducible expression vector pGEX-4T. GST–TCI fusion protein was expressed in *Escherichia coli* cells and purified by affinity chromatography on glutathione-agarose beads (Sigma) (Bazhan *et al.*, 2004).

Measuring antibody titres by ELISA

For ELISA, 96-well polystyrene microtitre plates (Greiner) were coated overnight at 4°C with 100 μl of 5 μg ml^{-1} purified recombinant TCI protein or inactivated HIV-1 virus (collection of SRC 'Vector', Russia) in carbonate buffer (pH 9.6) and blocked for 1 h at 37°C with the PBS containing 1% (w/v) BSA. The plates were then washed three times with the PBS containing 0.02% Tween 20 (PBST) and incubated with 100 μl of mouse serum for 1 h at 37°C Titration was performed at serum dilutions of 1:10 to 1:2000. The anti-mouse IgG antibodies conjugated to horseradish peroxidase (Sigma) were used as secondary antibodies and 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate. The optical density was measured in a Titertek at 450 nm. The data were analysed using Student's test.

ELISPOT assay

ELISPOT assays were performed as earlier described (Bazhan *et al.*, 2004). For this assay, 96-well microtitre plates (Millipore, USA) coated overnight with 100 μl per well of 5 μg ml^{-1} rat anti-mouse IFN-γ (BD PharMingen, USA) in PBS were washed with the PBS containing 0.25% Tween 20 and blocked with PBS containing 5% FBS for 2 h at 37°C. The plates were washed three times with the PBS containing 0.25% Tween 20, rinsed with the RPMI 1640 containing 10% FBS, and incubated in triplicate with 5×10^5 splenocytes per well in the reaction mixture with a volume of 100 μl containing one of the following peptides. Peptide 1 (EPFRDYVDRF), peptide 2 (DRVIEVVQGAYRAIR) and peptide 3 (CTEMEKEGKISKIGP) were used as specific antigens at a concentration of 1 μg ml^{-1}. Non-specific products were identified using the EHEC antigen. The plates were incubated overnight at 37°C in 5% CO_2 and washed with PBS. Biotinylated anti-mouse IFN-γ MAb (BD PharMingen) at a dilution of 1:500 were added to each well and incubated for 2 h at a room temperature. After washing, avidin-horseradish peroxidase (BD PharMingen) was added at a room temperature for 1 h. Individual IFN-γ-producing cells were detected as dark spots after a 10 min reaction with AEC (Sigma).

vided attenuated strain *S. enteritidis* E23. The work was supported by target funding of the anti-HIV-1 vaccine design according to the Order of the Government of the Russian Federation No. 1905.

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