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Induction of cytotoxic T lymphocyte response against Mycobacterial antigen using domain I of anthrax edema factor as antigen delivery system

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Abstract

We have investigated the efficiency of N-terminal 1–260 residues of Edema factor (EFn) as a delivery system for ESAT-6, an antigenic protein of *Mycobacterium tuberculosis* $H_{37}R_v$, into the cytosol of mammalian cells. The EFn.ESAT-6 recombinant protein was obtained by genetic fusion of EFn and ESAT-6 DNA. Our data shows that in the presence of PA, EFn.ESAT-6 fusion protein is internalized into the cytosol of antigen presenting cells, and the splenocytes produced both Th1 and Th2 cytokines *in vitro*. Further, EFn.ESAT-6 elicited effective cytotoxic T lymphocyte (CTL) response in an *in vitro* CTL assay. This study for the first time demonstrates that EFn can be used as a vehicle to deliver heterologous proteins of therapeutic importance.

Keywords: Edema factor; ESAT-6; Fusion protein; CTL; Cytokines

Humoral and cellular responses constitute the two main arms of immune system. One of the key functions of cellular immunity is to generate cytotoxic T lymphocytes (CTLs) for destruction of cells expressing intracellularly processed antigens on their surface. The CTLs recognize and kill tumor and other diseased cells, which display non-self peptides on their surface [1,2]. These peptides arise from various sources, such as infectious agents or aberrant expression of self-proteins, and mark defective cells for CTL recognition. Proteins within the cytosol are processed by multi-catalytic proteosome to generate small peptides, which are then displayed by class I major histocompatibility molecules (MHC-I) on the cell surface. Recognition of foreign peptide–MHC-I complexes by $CD8^+$ cells leads to activation of specific CTLs, which clear the defective cells expressing foreign peptides or harboring pathogen [1,2–5]. Activated CTLs lyse infected cell, secrete cytokines, proliferate and differentiate. Vaccines that prime such memory CTLs, provide protection to the host, upon subsequent exposure to similar antigen displaying cells [5].

Development of vaccines with the ability to generate specific CTLs is hindered due to paucity of delivery systems of antigenic CTL epitopes into the cytosol of host cells. Several approaches to this problem have been reported [1] including the use of attenuated viruses, intracellular bacteria, bacterial toxins, naked DNA, electroporation, heat shock protein, polycationic peptides, non-ionic triblock copolymer, and adjuvants [6–11]. Each of these methods have inherent problem of safety and/or efficiency. Non-infectious, non-toxic, modified bacterial toxins for delivery of heterologous proteins have been reported in the past [6,8,12–17].

Bacillus anthracis produces a bipartite exotoxin comprising of two toxins namely edema toxin (ET) and lethal toxin (LT). Both ET and LT contain a protective antigen (PA) component along with either edema factor (EF) or lethal factor (LF), respectively. Individually, PA, EF, and LF are non-toxic. PA mediates entry of EF and LF into the

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cytosol by specific receptor on the susceptible cells [18,19]. EF acts as Ca^{2+}/calmodulin dependent adenylate cyclase that causes edema by elevating cyclic amp levels abnormally in the host cells; [20] and LF, which has zinc metalloprotease activity, causes death by inactivating key molecules like MAPK kinase of signaling pathway [21,22]. Mutational studies in our laboratory have demonstrated that in the N-terminal half of EF, residues 136 VYYEIGK142 are critical for binding to PA and subsequent translocation into cytosol [23]. Additionally, the N-terminal domain I (EFn), (1–260 residues) lacks cytotoxicity, associated with the carboxyl terminal domain of EF, and therefore can be used safely for intracellular delivery of heterologous proteins for generation of CTL response.

Here, we report for the first time, utilization of EFn for delivery of ESAT-6, an antigen of *Mycobacterium tuberculosis* $H_{37}R_v$ into the cytosol of macrophages for induction of cell mediated immune response against ESAT-6.

Material and methods

Construction, expression, and purification of EFn.ESAT-6 fusion protein. EFn fusion protein containing ESAT-6 antigen was produced using expression vector pET23a (Novagen). The DNA sequence encoding EFn was amplified by PCR and cloned in pET23a vector using *Bam*HI and *Sac*I restriction sites. A *Kpn*I site was added by PCR amplification at 3′ end, just before *Sac*I site. A DNA sequence encoding ESAT-6 was amplified by PCR with *Kpn*I and *Sal*I sites at the 5′ and 3′ ends, respectively, and ligated to the above construct at *Kpn*1 site, such that ESAT-6 was fused at the C-terminal of EFn. This construct was sequenced to confirm the sequence.

pET23a construct containing EFn.ESAT-6 was transformed into competent cells of BL21 (DE3), Codon Plus strain of *Escherichia coli* and recombinant protein was obtained as follows. In brief, cultures were grown in LB medium containing ampicillin (100 μg/ml) and chloramphenicol (60 μg/ml) to an OD_{600} of 0.8. Protein expression was induced by 1 mM isopropyl α-D-thiogalactoside for 4 h. Cells were pelleted and EFn.ESAT-6 protein was purified using Ni–NTA affinity chromatography under denaturing conditions. The cell lysate containing the denaturant was mixed with 3 ml Ni–NTA slurry and loaded onto a column. Ni–NTA matrix was washed with 50 ml of denaturing buffer containing 8 M urea, followed by on-column renaturation of the protein using 8–0 M urea gradient. The protein was eluted with elution buffer containing 250 mM imidazole (pH 8.0). Purified fusion protein was analyzed on 12% SDS–PAGE and then dialyzed against 10 mM Hepes buffer containing 50 mM NaCl.

Purification of PA and LF. Recombinant PA and LF proteins were purified from culture supernatant of *E. coli* M15 cells as described previously [24,25].

Construction, expression, and purification of ESAT-6 protein. ESAT-6 protein was produced using the expression vector pQE30 (Qiagen). To produce ESAT-6, PCR amplified ESAT-6 gene was cloned using *Kpn*I and *Hin*dIII restriction sites at the 5′ and 3′ ends, respectively. The plasmid pQE30.ESAT-6 was transformed into *E. coli* M15 cells, and transformants were selected on plates containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml). *E. coli* M-15 culture containing plasmid pQE30.ESAT-6 was induced with 1 mM IPTG and ESAT-6 was purified as described above for EFn.ESAT-6.

Competitive inhibition of lethal toxin activity by EFn.ESAT-6. Binding of EFn.ESAT-6 to PA was tested on J774A.1 macrophage cell line by adding increasing concentrations of fusion protein in combination with PA (1 μg/ml) and LF (1 μg/ml) and incubating at 37 °C for 3 h. At the end of incubation period, cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye assay. MTT dissolved in RPMI 1640 medium was added to the cells at a final concentration of 0.5 mg/ml. Incubation was continued for additional 45 min to allow uptake and oxidation of the dye by viable cells. The medium was replaced by 100 μl of 0.5% SDS/25 mM HCl in 90% isopropanol, and plates were vortexed. Absorbance was read at 540 nm using a Microplate Reader (Bio-Rad).

Mice immunization. All the proteins used for immunization were tested for LPS, which was below 0.3 ng/μg of protein. Six- to eight-week-old female BALB/c (H-2^d) mice were obtained from Panacea Biotec Ltd. and maintained in pathogen free environment. A group of 10 BALB/c mice were injected intraperitoneally on day 0 and on day 14 with 2 μg of ESAT-6, EFn.ESAT-6, and PA+EFn.ESAT-6 in Phosphate buffer saline (PBS).

Isolation of splenocytes. Splenic effector cells were prepared by grinding spleens between frosted slides, followed by aspiration through 22-gauge needle to prepare single cell suspensions. Erythrocytes were lysed by ammonium chloride treatment. Remaining spleen cells were washed twice with complete RPMI medium and viability was determined by trypan blue exclusion. Splenocytes were suspended in complete RPMI medium containing 10% heat-inactivated FBS (Hyclone) and kept in CO_2 incubator for further use.

Cytokine ELISA. Mice were injected with PA+EFn.ESAT-6 and splenocytes were isolated after 7 days. Total splenocytes were cultured and stimulated *in vitro* by different antigens. Quantification of murine cytokines; IL-2, IL-12, IL-4, and IFN-γ present in the culture supernatant of activated splenocytes was determined using BD Opt EIA™ kits according to manufacture's protocol (Pharmingen).

Preparation of stimulator cells. For the preparation of stimulator cells, BALB/c (H-2^d) mice derived macrophage J774.1 cells were treated with the same antigen (1 μg/ml) as used for immunization [26]. ESAT-6 or EFn.ESAT-6 were either incubated with or delivered into J774A.1 cells by osmotic shock as described by Okada and Rechsteiner [27]. PA mediated delivery of EFn.ESAT-6 fusion was carried out as follows. Confluent J774A.1 macrophages in 75-cm^2-tissue culture flask were incubated with PA and EFn.ESAT-6 in RPMI-1640 glutamine minus medium for 4–5 h. The medium was removed, and extracellular protein/antigens were rinsed with complete RPMI 1640 medium. Macrophages were scraped off with a sterile rubber policeman and centrifuged at 200*g* for 5 min. Washed cells were suspended in 5 ml of complete RPMI 1640 medium with mitomycin C (35 μg/ml) and incubated at 37 °C for 45 min with 5% CO_2. Stimulator cells were washed by centrifugation four times with complete RPMI-1640 supplemented with 5% heat-inactivated fetal bovine serum. Trypan blue exclusion test was used to count macrophage cell numbers and to determine viability. As control of internalization, LF was also introduced by osmotic shock.

Preparation of effector cells. To obtain effector T cells, splenocytes from immunized mice and stimulator cells prepared with homologous antigen were mixed in 1:1 ratio and incubated in the presence of 30 U/ml murine recombinant IL-2 for 5 days at 37 °C with 5% CO_2. After 5-day incubation, cells were collected and live effector cells were obtained by removing dead cells by density gradient centrifugation over histopaque®-1119 (Sigma–Aldrich). Cell number and viability were determined by the trypan blue exclusion test.

Preparation of target cells. The protocol used to prepare different target cells was same as that for stimulator cells, except the mitomycin C step was omitted. J774A.1 cells were treated with 1 μg/ml each of PA, EFn.ESAT-6, and ESAT-6, PA+EFn.ESAT-6 for 4 h and washed twice with RPMI-1640 medium.

Colorimetric CTL assay. A previously described non-radioactive assay procedure [26,28] was followed with some modifications. This was preferred over the radioactive ^{51}Cr release CTL assay as being more sensitive and safe. Different combinations of effector and target cells were mixed at 10:1 ratio, respectively, and incubated for 16 h at 37 °C. After one wash with warm PBS (pH 7.2–7.4), 200 μl of 0.036% neutral red solution (prepared by diluting a 1% (wt/vol) stock solution to 0.036% in warm PBS (pH 7.2) just before use) was added to stain unlysed target cells. After 30 min, the cells were thoroughly washed and then lysed with 0.22 ml of 0.05 M acetic acid-0.05% sodium dodecyl sulfate solution. The amount of dye released was measured by taking optical density (OD) readings at