The Signal Peptide Sequence Impacts the Immune Response Elicited by a DNA Epitope Vaccine

Dimitrios Vatakis* and Minnie McMillan

Department of Molecular Microbiology and Immunology, University of Southern California, Keck School of Medicine, Los Angeles, California 90033

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We examined the effect of two leader sequences, one from a transmembrane molecule (H2-Ld) and another from a secreted molecule (rat KC chemokine), on the immunogenicity of DNA epitope vaccines. The chemokine leader enhanced vaccine immunogenicity, thus underscoring the importance of the leader sequence in DNA epitope vaccine design.

DNA vaccines have been shown to elicit strong humoral and cell-mediated immune responses in many animal models (2, 12, 13, 23). Studies suggest that the inoculated DNA is internalized by the local antigen-presenting cells (APCs), which then express and present the plasmid-encoded antigen to cytotoxic lymphocytes (CTLs) or T helper (Th) cells. Also, in vivo-transfected cells at the site of inoculation, such as keratinocytes and myocytes, can secrete the encoded antigen, which can be processed and presented by APCs (4, 6, 11, 23, 25).

The immune responses elicited by DNA vaccines have had varied results in different animal models, underscoring the need for optimization (1, 22, 23). An advantage of DNA vaccines is the ease with which one can introduce changes to qualitatively and quantitatively affect their immunogenicity (13, 23, 35). A number of approaches have been employed to enhance the efficacy of DNA vaccines, which include optimizing antigen processing (24) and presentation (5, 9, 17, 27, 29–31, 33, 37), using epitope-carrier protein fusion molecules (41), employing different delivery methods (18, 21, 22, 28, 34), and introducing immunomodulatory sequences in the plasmid vaccines (3, 8, 10, 13, 19, 22, 23, 40). In both full-length and minigenie DNA vaccines, leader sequences have been used to target the class I epitope to the ER more efficiently than would the other leader. We have analyzed and compared the immune responses elicited by the two vectors and demonstrate that the KC chemokine leader improved the immunogenicity of the DNA epitope vaccines.

To assess the role of the leader sequence in the DNA epitope vaccine immunogenicity, we constructed two vectors to be used in mouse immunizations (Fig. 1A). The first one, SAOLL, encodes a CTL epitope, HIV gp120 IIB (318–327) (A), which is targeted to the ER by the murine H-2Ld leader (S). The plasmid also contains a Th epitope, ovalbumin (OVA) (323–339) (O), which is targeted to the MHC II binding pathway by the dileucine lysosomal targeting motif (LL) (36). The second plasmid, RAOLL, is identical to SAOLL except that it encodes the rat KC chemokine (R) leader (Fig. 1A). Subsequently, DNA was prepared using the EndoFree Giga Prep kit (Qiagen, Valencia, CA). Three- to 5-week-old female BALB/cJ mice (The Jackson Laboratory, Bar Harbor, ME) were injected intradermally (i.d.) using a 26-gauge syringe with 200 μg of DNA in 80 μl of endotoxin-free phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO) and were boosted twice at 2-week intervals with the same amount of DNA. Two weeks later, mice were sacrificed by cervical dislocation and the spleens were excised from immunized and non-immunized (naive) mice. Splenocytes from individual mice were then in vitro stimulated with 1 μM gp120 peptide (317–326) for 5 days and used in a chromium release assay. Immunization of mice with the RAOLL vector elicited statistically significantly higher CTL responses than did immunization with the SAOLL vector (P < 0.01) (Fig. 1B). On average, we observed 20% higher specific lysis in mice immunized with the RAOLL vector than in mice immunized with the SAOLL vector. Thus, the use of the rat KC chemokine leader resulted in improved immune responses against the CTL epitope.

Furthermore, since our constructs encode a CD4 Th epitope, we carried out proliferation assays and enzyme-linked immunosorbent assays (ELISAs) to assess the impact of the rat KC chemokine leader on the OVA epitope responses in immunized mice. From the same experimental groups as those in Fig. 1B, splenocytes were stimulated with 40 μM ovalbumin (323–339), or myelin basic protein (MBP) (89–101) (negative-control) peptides for 72 h and used in a [3H]thymidine incor-

* Corresponding author. Mailing address: David Geffen School of Medicine at UCLA, Department of Medicine, Division of Hematology and Oncology, 615 Charles Young Dr. South, BSRB 173, M/C 736322, Los Angeles, CA 90095. Phone: (310) 206-2152. Fax: (310) 983-1067. E-mail: dvatakis@ucla.edu.

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FIG. 1. The use of the rat KC chemokine leader improved the immunogenicity of DNA epitope vaccines. (A) A schematic diagram of the vectors and signal sequences used in this study. Each vector encoded the murine Lδ or rat KC chemokine leader, the gp120 (318–327) CTL epitope, the ovalbumin OVA (323–339) epitope, a transmembrane domain, and the dileucine endosome targeting motif. The last two sequences facilitate the targeting of the OVA epitope to the MHC II antigen presentation pathway. (B) Mice immunized with RAOLL showed stronger CTL responses. Splenocytes from individual immunized and nonimmunized (naive) animals were used as effectors in a 51Cr release assay against P815 target cells pulsed with either gp120 (318–327) peptide (closed symbols) or the Dδ binding MPI peptide (negative control, open symbols). The data are from two (out of four) independent experiments using a total of 10 animals per group (except the naive group, in which we had a total of 6). Percentages in each group are depicted for the percent specific lysis at the 200:1 effector/target ratio. Statistical significance was determined by a two-tailed Student \( t \) test with \( P < 0.05 \) as significant. (C) Mice immunized with RAOLL showed increased proliferation following stimulation with the OVA epitope. The data are from the same groups as in panel B. The bar graph on the right represents the average cpm values from the dot plot. Statistical significance was determined by a two-tailed Student \( t \) test with \( P < 0.05 \) as significant. There was no statistical significance among the MBP groups. (D and E) IFN-\( \gamma \) and IL-2 secretion levels were higher in mice immunized with the RAOLL vector than in mice immunized with the SAOLL vectors and unimmunized mice (naive). Pooled splenocytes from each group were in vitro stimulated with ovalbumin (323–339) or MBP (89–101) for 72 h. Subsequently, supernatants were collected and used in a sandwich ELISA to detect IFN-\( \gamma \) (D) and IL-2 (E). The data are from one representative experiment (out of four).
ion assay. Mice immunized with the RAOLL vector exhibited stronger responses to the OVA epitope than did the SAOLL-immunized group, and the differences were statistically significant ($P < 0.01$) (Fig. 1C). In addition, we pooled splenocytes from immunized or naive mice and in vitro stimulated them with ovalbumin or MBP epitopes for 3 days. Supernatants were collected and used in a sandwich ELISA. Mice immunized with RAOLL exhibited higher levels of gamma

FIG. 2. The rat KC chemokine leader enhances immunogenicity of DNA epitope vaccines by increased antigen presentation and secretion. (A) Stable clones of P815 cells transfected with SAOLL and RAOLL were used as targets in a CTL assay. gp120-specific CTL lysed DV2A clones more efficiently than the DV2B ones. Nontransfected P815 cells pulsed with gp120 (318–327) were used as positive controls (P815/HIV) while targets pulsed with MPI were used as negative controls (P815/MPI). (B) A schematic diagram of the vectors used to assess secretion levels of gp120 under the murine H-2Ld and rat KC chemokine leaders. (C) An ELISA measuring the amount of gp120 secreted by the two leaders from stable clones generated from the 293T (squares), Bc10ME (triangles), and S194 (circles) cell lines transfected with pSA6H (open symbols) and pRA6H (closed symbols). A total of 18 clones were screened for the 293T and Bc10ME groups, and 8 clones were screened for the S194 group. Statistical significance for the data in this figure was determined by a two-tailed Student $t$ test with $P < 0.05$ as significant.
interference (IFN-γ) and interleukin-2 (IL-2) secretion than did the SAOLL-immunized mice (Fig. 1D and E). Based on our results, the use of the rat KC chemokine leader resulted in improved responses against the T$_H$ epitopes, which in turn can improve the quality of the CTL responses (37).

Furthermore, to determine whether the increased immunogenicity seen with the rat KC chemokine leader is due to improved antigen presentation, we transfected P815 cells with the SAOLL or RAOLL vector and generated stable clones that were used as targets in a $^{51}$Cr release assay. Effectors were generated from splenocytes of BALB/c mice stimulated with the gp120 peptide for 5 days as described above. P815 cells transfected with RAOLL were killed more efficiently by gp120-specific CTL than were the SAOLL clones (Fig. 2A). Similar patterns were seen in transiently transfected P815 cells (data not shown). Thus, the use of the rat KC chemokine leader resulted in improved antigen presentation.

In addition to improved antigen presentation, the rat KC chemokine leader could have impacted the immune response by increased protein secretion. To this end, we constructed two vectors, pSA6H and pRA6H, each encoding the 6×His-tagged synpg120 protein under either leader (Fig. 2B). The synpg120 gene was obtained from the pJR-FL synpg120 vector, and it is the gp120 MN gene optimized for mammalian codon usage (14). We transfected a series of cell lines with the above vectors and generated stably transfected clones. Supernatants from these clones were collected and screened by ELISA using Reacti-Bind metal chelation plates (high binding capacity) (Pierce, Rockford, IL). Briefly, samples were collected, cleared by centrifugation, and screened for secreted gp120 using goat anti-gp120 antiserum (PB1; NIH AIDS Reagent Program) and an alkaline phosphatase (AP)-conjugated rabbit anti-goat IgG antisera (Pierce). Cell lines transfected with the pRA6H vector released increased levels of gp120 protein (Fig. 2C). Similar patterns were seen in transiently transfected cells (data not shown). Based on the results above, the use of the KC chemokine leader resulted in higher levels of protein expression, which can enhance antigen presentation and vaccine immunogenicity.

Previous studies have examined the role of a leader sequence in minigene DNA vaccines with mixed results (5, 17). In this study, we compared two leader sequences, one derived from a transmembrane molecule, the murine H-2L^d, and the other derived from a secreted molecule, the rat KC chemokine (16), to determine the impact of the leader sequence on the immunogenicity of DNA epitope vaccines. Based on our results, the use of the rat KC chemokine leader resulted in improved responses against both the CTL and T$_H$ epitopes (Fig. 1). Furthermore, our data suggest that the improved responses were attributed to increased protein secretion (Fig. 2B and C) and higher antigen presentation (Fig. 2A). While the diverse nature of signal peptides is still under intense investigation, effective leader sequences share common features such as the presence of a hydrophobic core and low basicity (15, 20, 32, 38, 42). Both leader sequences used in our studies did share those features. However, in the rat KC chemokine leader, the amino acid residues near the cleavage site have smaller side chains (Ser, Thr) than those of the H-2L^d leader (Arg, Gln), which could account for its increased effectiveness (26, 43). In conclusion, our findings demonstrate that leader sequences can be used to rationally design minigene DNA vaccines.

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