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Production of Mouse Anti-CD4 Antibodies by DNA-Based Immunization

Watchara Kasinrerk, Niramon Tokrasinwit and Yaiyarat Piluk

SUMMARY **The intramuscular injection of plasmid DNA encoding an antigenic protein has been developed recently as a tool for immunization. DNA-based immunization was shown to generate immune responses against the encoded antigen in diverse animal species. In this report, we present the use of DNA-based immunization for the production of antibodies to CD4, a human leukocyte surface molecule. Mice were injected intramuscularly with eukaryotic expression vector containing cDNA encoding CD4 protein, termed CD4-DNA, and were subsequently assayed for anti-CD4 antibody production by indirect immunofluorescence. Sera collected from 2 of 3 inoculated mice reacted with CD4 expressing transfected COS cells and Sup-T1 cells. Anti-CD4 antibody activity was abolished by adsorption with CD4 molecule exppressing cells. $CD4^+$ cell depleted lymphocytes were also used to confirm the specificity of the anti CD4 antibodies present in immune serum. CD4-DNA immune serum reacted with approximately 1/3 of freshly isolated lymphocytes but to very few cells in the $CD4^+$cells-depleted preparation. CD4-DNA immunized sera was used to enumerate $CD4^+$cells in the peripheral blood of 6 healthy donors and 2 AIDS patients. The number of $CD4^+$ cells estimated by DNA immunized sera was very similar to estimates using standard anti-CD4 monoclonal antibody Leu3a. DNA-based immunization is therefore capable of raising antibodies to human leukocyte surface antigens. This technology may be useful for producing antibodies to other cell surface antigens in mice or other animals.**

The feasibility of directly injecting purified closed-circular plasmid DNA that encodes antigenic protein for producing both antibodies and cell mediated immune responses has recently been demonstrated.[1,2] This strategy can be defined as the physical delivery of an antigen encoding expression vector *in vivo* for the induction of antigen expression and the elicitation of specific immune responses. The DNA-based immunization technique has several advantages over conventional protein antigen immunization. DNA is simple to produce and purify than protein antigens and is highly stable.

Leukocyte surface antigens are molecules which are of critical importance for the function of human leukocytes. Antibodies raised against surface molecules have become a major tool in immunophenotyping and characterizing the structure of these surface molecules.[3] Highly pure antigen is required to produce polyclonal antibodies against a leukocyte surface molecule. DNA-based immunization therefore has the potential to be a novel alternative to standard antigen immunization. We report here an investigation into the production of antibodies to leukocyte surface antigens by DNA-based immunization. We found that injec-

From the Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand.
Correspondence : W. Kasinrerk

tion of plasmid DNA encoding human CD4 protein into mouse quadriceps muscle is capable of eliciting anti-CD4 antibodies. These antibodies reacted with either recombinant CD4 or native CD4 molecules. The DNA immunization technique is simple and rapid and produces antibodies capable of immunophenotyping leukocyte surface molecules.

MATERIALS AND METHODS

Cells and cell lines

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy donors or AIDS patients by Ficoll-Hypaque density centrifugation.[4] Sup T-1 cells, a human T cell line expressing high levels of surface CD4 protein,[5] were cultured in RPMI 1640 supplemented with 15% fetal calf serum (FCS; Gibco, Grant Island, NY), 40 μg/ml gentamycin and 2.5 μg/ml amphotericin B in a fully humidified atmosphere of 5% CO_2 at 37°C. COs cells were grown in Minimum Essential Medium (MEM; Gibco) containing 5% FCS and antibiotics at 37°C in a 5% CO_2 atmosphere.

Plasmid DNA for immunization

cDNA encoding CD4 protein was inserted into an eukaryotic expression vector πH3M (designated CD4-DNA), which was a kind gift from Dr. H Stockinger, University of Vienna, Vienna, Austria. πH3M is a high-efficiency eukaryotic expression vector containing a simian virus 40 (SV40) origin of replication and synthetic transcription units. The transcription units consist of a chimeric promotor composed of human cytomegalovirus AD169 immediate early enhancer sequences fused to the human immunodeficiency virus (HIV) long terminal repeat (LTR) sequences. A polylinker containing two *Bst*XI sites is inserted immediately downstream from the LTR sequence. The SV40 small tumor(t) antigen splice and early region polyadenylylation signals, which derived from pSV2, are placed downstream from the polylinker.[6]

Plasmid DNA preparations

Plasmid DNA was transformed into competent *E. coli* MC1061/p3 and the transformed bacteria were grown with vigorous shaking in 250 ml Terrific Broth per 1 liter flask. After overnight cultivation, cells were harvested and lysed by an alkaline lysis procedure.[7] DNA was then purified by cesium chloride-ethidium bromide density gradient ultracentrifugation.[7] The resulting DNA was phenol/chloroform-extracted, ethanol precipitated and resuspended in TE (10 mM Tris, 1 mM EDTA) pH 8.0 for transfection or immunization into mice. The concentration and purity of each DNA preparation was determined by OD260/280 reading.[7]

DNA immunization

Isolated plasmid DNA was injected weekly into the quadriceps muscle of both hind legs (100 μg per leg) of 4-8 week-old Balb/C female mice. Blood samples were collected from the retro-orbital venous plexus of ether-induced anesthetized mice prior to each DNA inoculation. Serum samples were then separated and stored at -20 °C.

DEAE-Dextran transfection of COS cells

Plasmid DNA encoding CD4 or CD8 proteins were transfected into COS cells using the DEAE-Dextran transfection method.[8] Briefly, $1x10^6$ COS cells were transfered to 6 cm tissue culture dishes (NUNC, Roskilde, Denmark) on the day before transfection. Cells were transfected in 2 ml of MEM containing 250 μg/ml DEAE-Dextran, 400 μM chloroquine diphosphate and 2 μg DNA. After 3 hours at 37 °C, the transfection mixture was removed and the cells were treated with 10% DMSO in PBS for 2 minutes at room temperature. Cells were cultured in MEM containing 5% FCS overnight, washed once, and recultured with the same medium for another 2 days to allow expression of CD4 or CD8 proteins.

Immunofluorescence analysis

The specificity of anti-CD4 protein antibodies was assessed by indirect immunofluorescence using FITC-conjugated sheep anti-mouse immunoglobulin antibodies (Dakopatts, Glostrup, Denmark). To block non-specific Fc receptor mediated antibody binding, tranfected COS cells, Sup T-1 cells or PBMC were incubated for 30 minutes at 4 °C with 10% human AB serum before staining. Blocked cells were then incubated for 30 minutes at 4 °C with various dilutions of tested antisera or anti-CD4 monoclonal antibody (MAb) (Leu 3a; Becton Dickinson, Sunnyvale, Ca) or anti-CD8 MAb (Leu 2a;

Becton Dickinson). After washing, cells were incubated with the FITC-conjugate for another 30 minutes. Membrane fluorescence was analyzed under a fluorescence microscope or flow cytometer (FACSCAN, Becton Dickinson). For flow cytometric analysis, individual populations of blood cells were gated according to their forward and side scatter characteristics.

Detection of anti-CD4 antibody activity and immunoadsorption

Serum samples from immunized mice were tested for anti-CD4 antibody activity by indirect immunofluorescence using either transfected COS cells or the Sup-T1 cell line. Stained cells were analyzed by fluorescent microscopy or FACSCAN. Immunoadsorption of anti-CD4 antibody from immunized serum was performed to confirm the specificity of anti-CD4 antibodies. Briefly, CD4-DNA immunized serum was incubated with $1x10^7$ Sup-T1 cells or CD4 or CD8 expressing transfected COS cells for 60 minutes at 4°C. After incubation, the cells were removed by centrifugation. Anti-CD4 antibody activity was then re-analyzed by using CD4 or CD8 expressing transfected COS cells or Sup-T1 cells by indirect immunofluorescence.

$CD4^+$ cell depletion

PBMC were treated with anti-CD4 MAb Leu3a (Becton Dickinson) at 4°C for 30 minutes. The treated cells were then mixed with sheep anti-mouse IgG-coated Dynabeads (M450; Dynal, Oslo, Norway). Cells that bound the iron conjugate were separated with a magnet in accordance with the recommended protocol (Dynal).

Enumeration of the percentage of $CD4^+$ cells in clinical specimens

PBMC were isolated from heparinized blood of healthy donors or AIDS patients by the standard Ficoll-Hypaque density centrifugation methed.[4] Isolated PBMC were stained with CD4-DNA immunized serum, non-immunized serum or anti-CD4 MAb Leu3a by indirect immunofluorescence. Membrane fluorescence was analyzed on a FACSCAN.

RESULTS

Large-scale preparation of CD4-DNA and expression of recombinant CD4 protein in COS cells

CD4-DNA was amplified in *E. coli* MC 1061/p3, and the plasmid DNA were isolated from transformed bacteria using the alkaline lysis procedure, and then purified by CsCl-EtBr gradients. The DNA yields were determined by OD 260/280 reading after completion of all the purification steps. In 3 lots of DNA preparations, the OD 260/280 ratios were between 1.8-2.0 The yields of purified plasmid DNA were approximately 3 mg/litre of bacteria.

The isolated plasmid DNA were then transfected into COS cells and analyzed for CD4 protein expression by indirect immunofluorescence. The CD4-DNA transfected COS cells showed strong positive reaction with anti-CD4 MAb Leu3a, but did not react with anti-CD8 MAb leu2a. These results indicated that the isolated CD4-DNA encodes CD4 protein and the encoded protein can be expressed on eukaryotic COS cells.

Production of anti-CD4 antibodies by CD4-DNA immunization

Three Balb/C mice were immunized with CD4-DNA at one-week intervals. For negative controls, mice were immunized with M6-DNA, which encodes a human leukocyte surface molecule,[8] or with πH3M vector lacking any expressible gene insert. The anti-CD4 antibody activity in sera was evaluated by indirect immunofluorescence using CD4-DNA and CD8-DNA transfected COS cells as antigens. Serum anti-CD4 antibody activity was detected in 2 of the 3 immunized mice injected with CD4-DNA (Table 1). Neither preimmune sera from the same mice nor control sera was reactive (Table 1.)

The reactivity of anti-CD4 antibodies was also investigated using Sup-T1 cell line. This cell line was positive with CD4-DNA immunized sera, but negative with preimmune sera (Fig.1). Anti-CD4 titers were 1:160 and 1:40.

Anti-CD4 antibody specificity

CD4-DNA immunized serum was adsorbed with either Sup-T1 cells, CD4-DNA transfected COS cells or CD8 -DNA transfected COS cells. The anti-CD4 antibody activity of adsorbed sera was then re-analyzed with CD4-DNA transfected COS cells, CD8-DNA transfected COS cells and Sup-T1 cells. As shown in Table 2, the activity of anti-CD4 antibodies was eliminated after adsorption with Sup-T1 or CD4-DNA transfected

Table 1. Anti-CD4 antibody activity in sera before and after DNA immunization

Mouse No.	Immunization	Serum	Immunofluorescent reactivity	
			CD4-COS[a]	CD8 -COS[b]
1	CD4-DNA	Pre-immunization	-	-
		Dose 1	-	-
		Dose 2	-	-
		Dose 3	+	-
		Dose 4	+	-
		Dose 5	+	-
		Dose 6	+	-
		Dose 7	+	-
		Dose 8	+	-
2	CD4-DNA	Pre-immunization	-	-
		Dose 6	+	-
		Dose 7	+	-
		Dose 8	+	-
3	CD4-DNA	Pre-immunization	-	-
		Dose 1	-	-
		Dose 2	-	-
		Dose 3	-	-
		Dose 4	-	-
		Dose 5	-	-
		Dose 6	-	-
		Dose 7	-	-
		Dose 8	-	-
4	Vector-DNA[c]	Pre-immunization	-	-
		Dose 6	-	-
		Dose 7	-	-
		Dose 8	-	-
5	M6-DNA[d]	Pre-immunization	-	-
		Dose 6	-	-
		Dose 7	-	-
		Dose 8	-	-

[a] COS cells transfected with CD4-DNA were strongly positive with anti-CD4 MAb Leu3a.

[b] COS cells transfected with CD8-DNA were strongly positive with anti-CD8 MAb Leu2a.

[c] Vector lacking any expressible gene insert.

[d] Plasmid DNA encoding M6 protein.[8]

COS cells. In contrast, anti-CD4 activity was still detected in serum after adsorption with CD8-DNA transfected COS cells.

Peripheral lymphocytes before and after $CD4^+$ cell depletion were used to confirm the specificity of anti-CD4 antibodies. As shown in Table 3, CD4-DNA immunized serum stained approximately 30% of cells in freshly isolated lymphocytes, but only about 4% in a $CD4^+$ cell depleted population. As a control, anti-CD4 MAb Leu3a was used, and a similar reaction pattern was found (Table 3.) Preimmune serum did not react with either the depleted or non-depleted population.

Enumeration of the percentage of $CD4^+$ cells in peripheral blood samples using CD4-DNA immunized serum

To further characterize the anti-CD4 antibody activity in CD4-DNA immunized serum, we used this serum for the enumeration of $CD4^+$ cells in peripheral blood. PBMC from 6 healthy subjects and 2 AIDS patients were stained with CD4-DNA immunized serum and anti-CD4 MAb Leu3a side by side. The stained cells were analyzed by flow cytometry. Both methods gave similar results (Table 4.) Non-immunized normal mouse serum was included in all experiments and gave no reaction (Table 4.).

DISCUSSION

Several investigators have demonstrated that the immunization of mice with a plasmid DNA vector

Table 2. Immunoadsorption of anti-CD4 antibody activity generated by CD4-DNA immunization.

Serum dilution	Adsorbed cells	Immunofluorescent reactivity[a]		
		CD4-COS[b]	CD8-COS[c]	Sup-T1
1:40	None	+	-	+
	Sup-T1	-	-	-
	CD4-COS[b]	-	-	-
	CD8-COS[c]	+	-	+
1:80	None	+	-	+
	Sup-T1	-	-	-
	CD4-COS[b]	-	-	-
	CD8-COS[c]	+	-	+

[a] Immunofluorescent reactivity was analyzed by fluorescent microscopy
[b] CD4-DNA transfected COS cells.
[c] CD8-DNA transfected COS cells.

Table 3. Percentage of $CD4^+$ cells in peripheral blood lymphocytes before and after $CD4^+$ cell depletion determined by staining with CD4-DNA immunized serum and anti-CD4 MAb Leu3a

Donor No.	Anti-CD4 MAb Leu3a		DNA immunized serum[a]		Pre-immune serum	
	Before[b]	After[c]	Before	After	Before	After
1	33[d]	4	31	4	1	1
2	25	2	28	2	0.7	0.3

[a] Serum obtained from CD4-DNA immunized mouse.
[b] Before $CD4^+$ cell depletion
[c] After $CD4^+$ cells depletion.
[d] Percentage of $CD4^+$ cells was determined by flow cytometric analysis.

containing genes results in the induction of immune responses to the encoded proteins.[1,2] The use of DNA-based vectors as an alternative to antigen immunization is a novel strategy, now under development and evaluation. Plasmid vectors containing several genetic elements are required to drive the intracellular expression of the foreign gene insert. These genetic elements include (i) a transcriptional promotor, (ii) an optional enhancer element to augment gene expression, (iii) the foreign gene encoding an antigenic gene product, and (iv) RNA-processing elements, primarily a polyadenylation signal and an optional intron elements.[2] In addition, the plasmids should contain two bacterium-specific genetic sequences to allow large scale production of DNA, *i.e.* an antibiotic selectable marker to permit the identification and isolation of bacterial cells successfully transduced with the gene of interest, and a bacterial origin of replication to facilitate large scale amplification of the plasmid DNA within this host cell. Once the DNA enter the mammalian cells, the encoded proteins are expressed through normal cellular transcription and translation mechanisms. Immunization with DNA-based plasmids has been attempted successfully in various species including mice, chicken, ferrets, cattle, and non-human primates by various routes of administrations.[9-15] Most experiments, however, have been conducted with DNA.

cDNA encoding CD4 protein constructed in the eukaryotic expression vector πH3M was used to produce antibodies to leukocyte

Table 4. Determination of the percentage of $CD4^+$ lymphocytes using anti - CD4 MAb Leu3a and CD4-DNA immunized serum

Subject No.	% Positive cells in lymphocytes		
	Anti-CD4 MAb Leu3a	DNA immunized serum[a]	Non-immunized serum
1	39.1	39.7	0.9
2	33.5	33.1	1.0
3	29.2	30.4	2.0
4	31.5	31.4	0.9
5	26.6	32.5	1.9
6	37.5	39.1	1.2
7[b]	5.0	5.4	0.5
8[b]	0.5	0.5	1.1

[a] Serum obtained from CD4-DNA immunized mouse.

[b] Subject Nos. 7 and 8 were AIDS pateints and by standard Simutest flow cytometry the percentage of $CD4^+$ lymphocytes were 7 and 2, respectively.

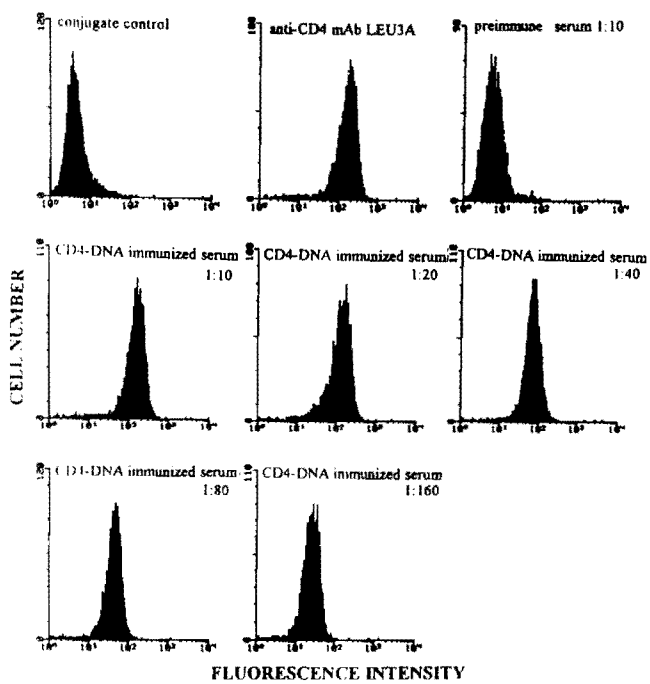

Fig. 1. Immunofluorescent analysis of the reactivity of anti-CD_4 antibodies with the Sup-T1 cell line. Sup-T 1 cells were stained with either anti-CD^4 MAb Leu3a, pre-immune serum or CD^4-DNA immunized serum. The intensity of fluorescence was measured by FACSCAN with logarithmic amplification.

surface antigens. The transcription units presented in the πH3M vector meet the criteria for plasmid vectors used in DNA-based immunization. This study demonstrates that mice inoculated with πH3M containing encoding CD4 protein induces anti-CD4 antibody; 2 of 3 inoculated mice generated anti-CD4 antibodies detectable by immunofluorescence. Anti-CD4 antibodies bound either recombinant CD4 proteins which were expressed on transfected COS cells, or native CD4 proteins on lymphocytes and Sup-T1 cells. Direct intramuscular injection of plasmid DNA has been widely used to induce antibody production, due to its simplicity and effectiveness. However, a large quantity of DNA is required (approximately 100-300 μg/inoculation).[12,13,16-18] Alternatively, delivery of DNA-coated gold beads into epidermis by a biolistic device has been shown to generate immune responses.[10,19,20] This biolistic transfection required less DNA than intramuscular inoculations.

CD4-DNA immunized serum was used to count $CD4^+$ cells in peripheral blood and gave comparable results to standard methods in 6 healthy PBMC and 2 AIDS patients. A reduction in circulating $CD4^+$cells is characteristic of AIDS patients,[21] and was found in our 2 patiens using both CD4-DNA immunized serum and anti-CD4 MAb Leu3a.

The direct introduction of genes into mouse muscle and its expression *in vivo* may allow the processing of gene products and lead to the presentation of an effective target antigen. This DNA-based immunization strategy is simple and rapid. To our

knowledge, production of antibody to the human leukocyte surface antigen using DNA immunization technology has not been previously reported. Anti-CD4 antibodies, as we demonstrated here, can be used to determine cell surface molecules, and have many other potential applications.

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