Arch. Pharm. Res. Vol.21, No.5, pp. 537-542, 1998

Vaccination with an Ovalbumin/Interleukin-4 Fusion DNA Efficiently Induces Th2 Cell-Mediated Immune Responses in an Ovalbumin-Specific Manner

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(Received June 1, 1998)

To more effectively drive immune responses toward antigen-specific T helper type 2 (Th2) cell-mediated responses, we constructed a mammalian expression vector (pOVA/IL4) carrying a fused gene in which the ovalbumin (OVA) cDNA was covalently linked to murine interleukin-4 (IL-4) cDNA. A biologically active OVA/IL4 protein was expressed by the transfected COS cells with the pOVA/IL4 DNA, as demonstrated by Western blotting and cytokine bioassay. Intramuscular injection of BALB/c mice with the pOVA/IL4 DNA increased both the production of OVA-specific IL-4 by $CD4^+$ T cells and the ratio of anti-OVA IgG1 to anti-OVA IgG2a isotypes, while the injection with the pOVA DNA alone, or with the mixture of the pOVA and pIL4 DNA did no or little increase. Furthermore, the OVA-specific, Th2 cell-mediated immune responses were significantly enhanced by multiple injections with the pOVA/IL4 DNA. These studies indicate that the direct linkage of an OVA gene to an IL-4 gene in the expression plasmid confines the effects of IL-4 to the OVA-specific cells, efficiently driving the immune response toward OVA-specific, Th2 cell-mediated responses.

Key words : Ovalbumin, Interleukin-4, DNA vaccine, T helper cell

INTRODUCTION

A major advance in understanding the regulation of specific immune responses was the identification of two subpopulations of $CD4^+$ T helper (Th) lymphocytes, Th1 and Th2 cells, based on their mutually exclusive production of cytokines (Constant and Bottomly, 1997). Th1 cells produce interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β), whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13. Th1 cells are the principal effectors of cell-mediated immunity and of delayed-type hypersensitivity reactions and they also stimulate the production of IgG2a antibody isotype. On the other hand, Th2 cells induce the production of IgG1 and IgE antibody isotypes via IL-4, favor the differentiation and activation of eosinophil, and are capable of inhibiting some macrophage functions (Mosmann *et al.*, 1991).

Recent studies indicate that the ratio of two Th cell types, Th1 and Th2, is closely correlated with the outcome of many diseases (Romagnani, 1996a; Romagnani *et al.*, 1997). Polarized Th1-type and Th2-type responses play different roles in protection, Th1 being effective in the defense against intracellular pathogens and Th2 against intestinal nematodes. Moreover, Th1 responses predominate in organ-specific autoimmune disorders, acute allograft rejection, unexplained recurrent abortions and in some chronic inflammatory disorders of unknown etiology. In contrast, Th2 responses predominate in Omenn's syndrome, transplantation tolerance, chronic graft *versus* host disease, systemic sclerosis. Especially, in allergic diseases, Th2 cells are preferentially activated, and the IL-4 and IL-5 produced by the Th2 cells cause increased IgE production and eosinophilia, respectively.

The nature of Th1 or Th2 polarizing signals is not yet fully understood. However, the factors that seem to play a role in driving naive $CD4^+$ T cells toward Th1- or Th2-dominated populations are the type and the amount of antigen as well as the density and affinity of the peptide ligand, the nature of antigen-presenting cells and of their co-stimulants, and hormones released into the microenvironment, and genetic background of the T cell (Romagnani, 1996b). Most importantly, the cytokines that are present in the environ-

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ment of the $CD4^+$ T cell at the time it encounters the antigen regulate the differentiation of Th cells into either Th1 or Th2 subsets. IL-12 and IFN-γ promote Th1 differentiation (Constant and Bottomly, 1997), while IL-4 plays a key role in the differentiation of the precursor $CD4^+$ T cells toward a Th2 phenotype (Gross *et al.*, 1993). In previous studies, we demonstrated that the OVA/IL-12 or OVA/IFN-γ fusion vaccines efficiently drived immune responses toward Th1 cell-mediated response in an antigen-specific manner (Kim *et al.*, 1997; Lim *et al.*, 1998).

In this study, to investigate further the role of antigen-cytokine fusion constructs as a vaccine for controlling Th cell-mediated immune responses, and to drive immune responses toward antigen-specific Th2 responses by DNA-based immunization, we constructed a mammalian expression plasmid (pOVA/IL4) that contained a fused gene of the ovalbumin cDNA and murine IL-4 cDNA, linked by a spacer of 6 amino acids-encoded nucleotides. Our results showed that the injection with the pOVA/IL4 DNA induced OVA-specific, Th2 cell-mediated immune responses, while the injection with the pOVA alone or the mixture of the pOVA and plL4 did not. These results demonstrated that the direct linkage of an IL-4 cDNA and an OVA cDNA efficiently confines the effects of IL-4 to the OVA-specific cells.

MATERIALS AND METHODS

Cell lines and mice

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics. HT-2 cells were maintained in RPMI 1640 supplemented with 10% FBS and antibiotics (Gibco BRL, Grand Island, NY). Both cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). Female BALB/c ($H\text{-}2^d$) mice were purchased from the B & K Universal Group Limited (Fremont, CA), and were used at 6 to 10 weeks of age.

Cytokines, antigens and antibodies

Murine recombinant IL-4 and recombinant IFN-γ were obtained from Genzyme Co. (Cambridge, MA). Ovalbumin (OVA) was obtained from ICN Biomedicals (Montreal, PQ). Two anti-OVA mAbs (Dr. Umetsu, Stanford University, Stanford, CA), 6C1 (IgG1) and 3A 11 (IgG2a), were used as standards for isotype-specific ELISA. Polyclonal mouse anti-OVA antibodies were prepared from sera of immunized mice after repetitive injections of OVA in CFA, followed by OVA in IFA. HRP-labelled goat antibodies to mouse IgG, IgG1, and IgG2a were purchased from Southern Biotechnology Associates Inc. (Birmingham, AL).

Construction of an expression plasmid carrying an OVA/IL4 hybrid gene

A mammalian expression plasmid containing a SV 40 origin of replication and designed for expression of immunoglobulin genes (donated from Dr. Reff) (Reff *et al.*, 1994), was modified to eliminate most of the immunoglobulin (Ig) coding regions as well as the neomycin resistance gene. The pOVA/IL4 DNA was constructed by first inserting the OVA gene in frame with the human Ig kappa leader sequence, to allow for secretion of the translated protein. The IL-4 gene was inserted downstream of the OVA gene, separated by a spacer that encoded for the amino acids S, S, G, G, G, and G. The OVA and IL-4 genes were cloned by PCR from previously cloned cDNA constructs using primers containing the desired restriction sites; OVA primers (DraIII-OVA 5' GGCCACGATGTGGCTCCATCGG 3'; OVA-XhoI 5' CCTCTCGAGGGGGAAACACATCT 3') and IL-4 primers (XhoI-IL4 5' CGCCCTCGAGCGGACATATCCACGGA 3'; IL4stop-BamHI 5' CGCCGGATCCCTACGAGTAATCCATTTG 3'). As controls, the pOVA and plL4 were constructed by PCR using primers containing a stop codon at the 3' primer of each gene; OVA primers (DraIII-OVA 5' GGCCACGATGTGGCTCCATCGG 3'; OVAstop-BamHI 5' CGCCGGATCCCTAGGGGGAAACACATCT 3') and IL-4 primers (DraIII-IL4 5' GGCCACGATGTGGCCATATCCACGGA 3'; IL4stop-BamHI 5' CGCCGGATCCCTACGAGTAATCCATTTG 3'). The constructed plasmids were, respectively, electroporated into *E. coli*, and purified from large-scale cultures by alkaline lysis and cesium chloride density gradient centrifugation (Coligan *et al.*, 1991). The endotoxin level of the purified plasmids was <20 EU/mg DNA, as detected using the limulus amebocyte lysate (LAL) assay kit (BioWhittaker, Walkersville, MD).

Transfection and SDS-PAGE

COS-7 cells were transfected with the pOVA/IL4 or pOVA DNA using DEAE-dextran in a standard protocol (Coligan *et al.*, 1995). Supernatants from the transfected cells were harvested after 3 days, and secreted proteins were immunoprecipitated using anti-OVA antibody-conjugated CNBr-activated Sepharose 4B resin (Sigma Chemical Co., St. Louis, MO). The resins were washed 3 times with 0.1% Tween 20 in Tris buffer (pH 8.0), and then the precipitated proteins were eluted in SDS-PAGE sample buffer and analyzed on SDS-PAGE. The SDS-PAGE was performed using gradient gels in a Mini-Protein II gel apparatus (Bio-Rad, Richmond, CA). Proteins were stained with Coomassie brilliant blue.

Intramuscular injection of plasmid DNA

Mice were injected intramuscularly (i.m.) with 150 μg DNA in 100 μl of 0.85% normal saline into each quadricep muscle by using a 28-gauge insulin syringe. The quadricep muscles were visualized by making an 1 cm incision in the skin with a microdissecting scissor. The injection depth of the needle was adjusted to 2 mm by using a steel collar as described previously (Wolff *et al.*, 1990).

Purification of CD4 T$^+$ cells and *in vitro* stimulation

BALB/c mice were injected i.m. with the pOVA or pOVA/IL4 DNA as indicated in the figure legends, and spleens were obtained from the immunized mice and single cell suspensions were prepared (DeKruyff *et al.*, 1995). The CD4$^+$ T cells from the spleen cell suspensions were enriched by negative selection using Magnetic Activated Cell Sorting (MACS) with a cocktail of biotinylated anti-mouse CD8, I-A, B220, and Mac-1 antibodies as previously described (Miltenyi, Sunnyvale, CA) (Penshaw *et al.*, 1995). More than 98% of the isolated cells were CD4$^+$ T cells as analyzed by cytofluorometry. The purified CD4$^+$ T cells (5×10^5 cells/well) were distributed into each well of 96-well plate and incubated *in vitro* with 100 μg/ml of OVA or KLH for 4 days (IFN-γ and IL-4 production assay).

Cytokine assays

The quantities of IFN-γ and IL-4 in the culture supernatants of CD4$^+$ T cells were determined by a sandwich ELISA using mAbs specific for each cytokine (Genzyme Co., Cambridge, MA). The biological activity of IL-4 in the OVA/IL-4 protein was determined by the ability to stimulate the proliferation of HT-2 cells as described previously (Coligan *et al.*, 1995). Recombinant mouse IFN-γ and IL-4 (Genzyme Co.) were used as a standard.

Determination of anti-OVA antibody isotypes

Mice were bled during the course of experiments, and the amounts of OVA-specific antibody isotypes in the sera were measured by an ELISA using mAbs specific for each isotype. Briefly, ELISA plates were coated with 100 μl of 5 μg/ml OVA per well. After coating, serial dilutions of sera were added to the plates and incubated overnight at 4°C. Anti-OVA IgG1 (6C1), anti-OVA IgG2a (3A11), and anti-OVA total IgG antibodies were used as standards for quantitation of each IgG subclass. After washing, HRP-labelled anti-mouse IgG1, IgG2a, and IgG were, respectively, added and incubated for 2 hr at room temperature. After additional washing, O-phenylenediamine (OPD) substrate was added and developed for 10 min, and the O.D. was determined at 492 nm in a Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA).

RESULTS

Expression of a biologically active OVA/IL4 protein by the transfected COS cells with pOVA/IL4 DNA

The expression plasmid carrying an OVA/IL4 fused gene (pOVA/IL4) was constructed, as described in the Materials and Methods. The pOVA/IL4 was tested for its ability to produce a recombinant OVA/IL4 fusion protein by transient transfection of COS-7 cells. As a control, the plasmid carrying an OVA gene (pOVA) was also constructed and the expression of recombinant OVA protein by the transfected COS-7 cells was determined. The recombinant OVA/IL4 protein secreted by the transfected COS-7 cells was pu-

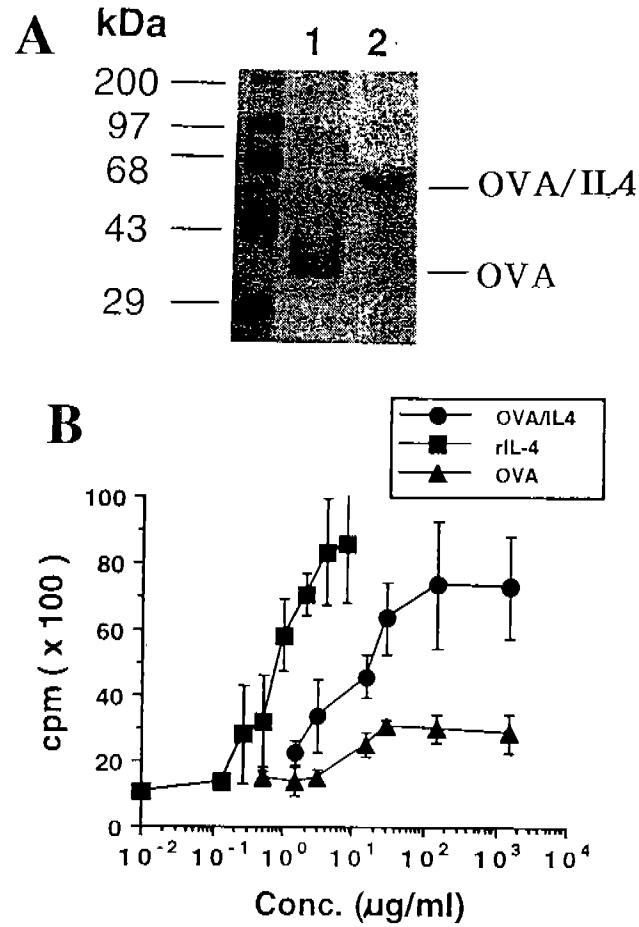

Fig. 1. Expression of a recombinant OVA/IL4 protein by the COS cells transfected with the pOVA/IL4 DNA. (A) SDS-PAGE of the OVA/IL4 protein expressed by the transfected COS cells. The OVA or OVA/IL4 proteins were immunoprecipitated with anti-OVA mAb from the supernatants of the transfected cells, and electrophoresed. (B) Biological activity of an OVA/IL4 protein produced by the transfected COS cells, as determined by the ability to stimulate the proliferation of HT-2 cells.

rified by anti-OVA immunoprecipitation, and analyzed by SDS-PAGE and Western blotting using anti-OVA mAb, showing that the supernatant contained a protein of the appropriate size for the OVA/IL4 fusion construct (Fig. 1A). Furthermore, the COS cell-derived OVA/IL4 protein stimulated the proliferation of IL-4-dependent HT-2 cells in a dose-dependent manner, suggesting that the recombinant OVA/IL4 protein was biologically active (Fig. 1B).

Formation of anti-OVA antibody in mice immunized with the pOVA/IL4 DNA

To examine the effect of the pOVA/IL4 DNA on the production of OVA-specific antibody, BALB/c mice were immunized i.m. with 150 μg pOVA/IL4, or with 150 μg pOVA as a control. Mice were bled 2, 4, 8, 12, 16 weeks after the injection, and antibody responses in the sera were determined by an OVA-specific antibody ELISA. As indicated in Fig. 2, the titers of anti-OVA antibody in the immunized mice were continuously increased until 12 weeks after the single injection with the DNAs although the levels were relatively low (less than 5 μg/ml). BALB/c mice immunized with either the pOVA or pOVA/IL4 DNA showed similar time courses of anti-OVA antibody production, and the levels between two groups were not significantly different.

Th2 cell-mediated immune responses induced with DNA immunization with the pOVA/IL4

To examine whether the injection with the pOVA/IL4 DNA induces Th2 immune response in an OVA-specific manner, the mice were immunized i.m. with 150 μg of the pOVA/IL4 or pOVA and, 8 weeks after the injection, the concentrations of OVA-specific IgG2a and OVA-specific IgG1 antibody isotypes in the immunized mice were determined. We included a group of mice injected with a mixture of the pOVA (150 μg) and pIL4 (150 μg) to investigate the importance of the direct linkage between the OVA and the IL4 in the pOVA/IL4 plasmid. As shown in Fig. 3, multiple injections with the pOVA/IL4 DNA dramatically increased the amounts of anti-OVA IgG1, leading to higher ratio of [anti-OVA IgG1]:[anti-OVA IgG2a] than that induced with the pOVA, or with the mixture of the pOVA and pIL4. The multiple injections with the mixture of the pOVA and pIL4 also increased the ratio of [anti-OVA IgG1]:[anti-OVA IgG 2a]. However, the ratio, in this case, was greatly lower than that induced with the pOVA/IL4 fusion DNA vaccination. Therefore, the linkage between two genes of the OVA and IL-4 is required for efficiently inducing Th2 cell-mediated immune responses.

We next examined whether the multiple injections with the pOVA/IL4 DNA enhanced OVA-specific, Th2 cytokine profile from the $CD4^+$ T cells. The mice were immunized i.m. with 150 μg of the pOVA or pOVA/IL4, or with a mixture of pOVA and pIL4 at different times and, 2 weeks after the last injection, the levels of IFN-γ and IL-4 produced by splenic $CD4^+$ T cells were determined after *in vitro* stimulation with 100 μg OVA. As indicated in Fig. 4, injections with the pOVA/IL4 DNA increased OVA-specific IL-4 production, leading to higher ratios of [IL-4]:[IFN-γ] than those induced with the pOVA, or with the mixture of the pOVA and the pIL4 DNA.

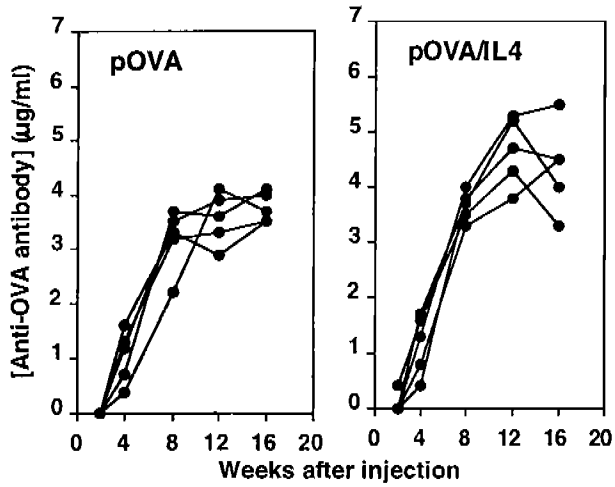

Fig. 2. Time course of anti-OVA antibody production in mice immunized with the pOVA/IL4 DNA. BALB/c mice (5 per group) were injected i.m. with 150 μg of the pOVA/IL4 or pOVA DNA, and the mice were bled at 2, 4, 8, 12, 16 weeks after the vaccination. Each line represents the titers of an individual mouse.

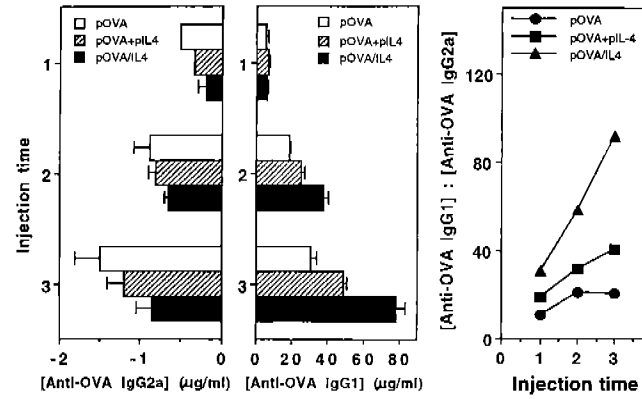

Fig. 3. Production of anti-OVA isotypes in BALB/c mice induced by vaccination with the pOVA/IL4 DNA. BALB/c mice were injected i.m. with 150 μg pOVA/IL4 or 150 μg pOVA DNA, or with a mixture of 150 μg pOVA and 150 μg pIL4 (one, two or three weekly injections). Eight weeks after the last injection, the levels of anti-OVA antibody isotypes in the sera were quantitated by isotype-specific ELISAs. The values represent the mean±SD of 5 mice. These data are a representative of two similar experiments.

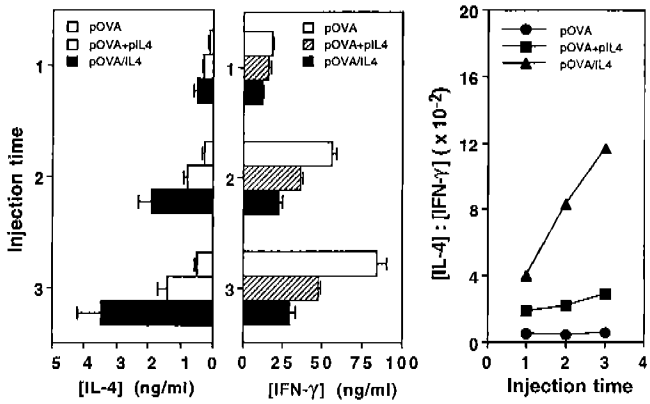

Fig. 4. Induction of OVA-specific Th2 cytokine profile from the $CD4^+$ T cells by vaccination with the pOVA/IL4 DNA. BALB/c mice were injected i.m. with 150 μg pOVA/IL4 or pOVA DNA, or with a mixture of 150 μg pOVA and 150 μg pIL4 (one, two or three weekly injections). Two weeks after the last injection, the production of IFN-γ and IL-4 by the $CD4^+$ T cells were determined after the stimulation of $CD4^+$ T cells *in vitro* with 100 μg OVA. The values represent the mean±SD of three separate experiments.

DISCUSSION

In this report we have shown that the linkage between IL-4 and OVA genes confined the effect of IL-4 to the OVA-specific cells, leading to Th2-dominated immune responses in an OVA-specific manner, when the mice were intramuscularly immunized with the pOVA/IL4 DNA. The Th2 response was efficiently enhanced by multiple injections with the pOVA/IL4 DNA. The induced Th2 responses were characterized by high titers of OVA-specific IgG1 antibody and by high levels of IL-4 production from the OVA-specific $CD4^+$ T cells. In contrast, the injection with a simple mixture of the pIL4 and the pOVA DNA also increased OVA-specific immune responses which, however, were no or little polarization toward a Th2 profile, as seen by the ratios of [anti-OVA IgG1] : [anti-OVA IgG 2a] in the immunized mice and of [IL-4] : [IFN-γ] produced from $CD4$ T^+ cells. Thus, the effects of IL-4 were efficiently confined to the OVA-specific cells by the covalent linkage of the IL-4 to the OVA.

DNA immunization has been proven to be effective to elicit protective immune response to a variety of pathogens from viruses to parasites. It seems that efficient uptake of the plasmid vector without any carrier molecule is due to intrinsic characteristics of straited muscles (Danko and Wolff, 1994). Whitten and Yokoyama (1996) reported that proteins expressed by DNA vaccines induced both local and systemic immune responses. Our data suggested that the secretion of the intact fusion protein by resident tissues for uptake by antigen-presenting cells occurred since the expression of the OVA/IL4 protein by the transfected COS-7 cells with the pOVA/IL4 DNA was confirmed and the OVA/IL4 DNA vaccination demonstrated *in vivo* the biological effects of IL-4. Furthermore, several factors including the identity of the antigen and the route of DNA adminstration have been suggested to determine the types of immune response following DNA immunization (Pertmer *et al.*, 1996; Yokoyama *et al.*, 1997). In the study we demonstrated that the direct linkage of a Th2-driving cytokine, IL-4, to an antigen could control the immune responses toward a Th2 profile. Multiple injections with the antigen/IL4 fusion DNA might increase the *in vivo* population of the transfected cells with the injected DNA and the expression level of the fusion protein, leading to drive the uncommitted and/or memory $CD4^+$ T cells toward a Th2 profile. Others reported that muscle degenerating agents such as bupivacaine and cardiotoxin were used 3~6 days before DNA vaccination to facilitate DNA uptake, leading to increase the expression of the inoculated gene up to several fold (Wang *et al.*, 1993; Wells, 1993). The mechanism by which the OVA/IL4 DNA induces OVA-specific, Th2-dominated response compared with the nonpolarization of Th response by the mixture of pOVA and pIL4 is not clear.

One possiblity is that the expressed OVA/IL4 protein may efficiently induce OVA-specific, Th2 immune responses by increasing the *in vivo* half-life of IL-4 activity in the OVA/IL4 fusion protein compared with free recombinant IL-4. Gillies *et al.* (1993) reported that either IL-2 or GM-CSF fused to antibodies had a prolonged half-life in serum compared with the cytokine alone. However, our co-immunization experiments demonstrated much more absolute dependence on fusing the cytokine to the antigen for inducing antigen-specific, Th2 immune responses. Immunization with the mixture of the pOVA and pIL4 also induced OVA-specific immune responses which, however, were not polarized toward either Th1 or Th2 profiles. Therefore, the linkage between the OVA and the IL-4 allows the Th2-driving effect of IL-4 to localize sites in immune organs where OVA-specific cells are being activated. The presence of cytokines in the microenvironment of antigen-specific immune responses gives advantages in T cell immunobiology and in cancer therapy, since direct injection of cytokine gene-containing vectors into tumor cells or injections of cells secreting specific cytokines resulted in tumor regression and acquisition of systemic antitumor immunity (Gilboa, 1996). The generality of this approach to various cytokines and other antigens remains to be examined. In addition, experiments should be done to determine whether the pOVA/IL4 DNA can convert the established Th1- to Th2-immune responses.

In conclusion, the OVA/IL4 fusion DNA can induce OVA-specific, Th2 cell-mediated immune responses,

compared with the Th response induced with the simple mixture of the pOVA and the pIL4. These studies suggest that the direct linkage of the IL-4 to the OVA can confine the effect of IL-4 to OVA-specific cells and, therefore, an antigen/IL4 plasmid may be beneficial in the treatment of diseases caused by undesired Th1 dominated responses, including autoimmune diseases and certain parasitic infections.

ACKNOWLEDGMENTS

We thank Drs D. T. Umetsu and H. T. Maecker, Stanford University for providing valuable reagents. This work was supported by a grant from the Korea Science and Engineering Foundation (971-0705-039-2) and in part from the Korean Research Foundation made in the Program Year 1996 to T. S. Kim.

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