Covalent Linkage of IL-12 and Ovalbumin Confines the Effects of IL-12 to Ovalbumin-specific Immune Responses

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In order to direct the form of the immune response in an antigen-specific manner, we constructed a fusion protein (OVA/IL12) that contained the T cell-dependent antigen, ovalbumin (OVA), covalently linked to murine interleukin-12 (IL-12). The OVA/IL12 protein was produced in a baculovirus expression system and was purified by anti-OVA immunoaffinity chromatography. The purified OVA/IL12 protein displayed potent IL-12 bioactivity in an IL-12 proliferation assay. BALB/c mice immunized with the OVA/IL12 protein produced increased quantities of anti-OVA IgG2a antibody compared with mice immunized with recombinant OVA alone. Lymph node cells from the immunized mice with the OVA/IL12 protein produced large amounts of IFN-γ when restimulated in vitro with OVA, while those from mice immunized with the OVA protein produced little or no IFN-γ. In contrast, immunization with a mixture of OVA and free recombinant IL-12 also induced IFN-γ production, which was not OVA-specific. These studies indicate that the OVA/IL12 fusion protein can induce OVA-specific, Th1-dominated immune responses, and that the covalent linkage of OVA and IL-12 confines the effect of IL-12 to OVA-specific cells.

Key words : Interleukin-12, Ovabumin, Th cell, Interferon-γ, Antigen-specific, Allergy

INTRODUCTION

Regulation of immune responses by CD4^{+} T cells is mediated by antigen-induced production of cytokines. Naive CD4^{+} T cells have limited cytokine responses and, therefore, their regulatory capacity and effector function are limited (Weinberg et al., 1990; Paul and Sedar, 1994). Antigen-driven activation of CD4^{+} T cells leads to their differentiation into two major subsets, Th1 and Th2, that are characterized by their function and cytokine secretion pattern. Th1 cells selectively secrete IL-2, IFN-γ and TNF-β, and regulate cell-mediated immunity characterized by the production of complement-fixing and opsonizing antibodies such as IgG2a. Th2 cells produce IL-4, IL-5 and IL-10, which are involved in development of humoral immune responses including expression of IgG1 and IgE antibodies. These two CD[+] T cell populations regulate each others' function through the antigonistic activity of their respective cytokines.

The commitment of Th cells to Th1 or Th2 cells is of crucial importance with respective to susceptibility or resistance to particular infections, or to autoimmune

diseases and allergic diseases. For example, in allergic diseases and parasite infections, especially helminth infections, Th2 cells are preferentially activated, and the IL-4 and IL-5 produced by the Th2 cells cause increased IgE production and eosinophilia, respectively. A number of factors determine whether an immune response will be dominated by Th1 or Th2 cells. Most importantly, the cytokine microenvironment in which the initial antigen priming occurs is responsible for influencing differentiation of CD4^{+} T cells into either Th1 or Th2 cells. IL-12 drives differentiation of Th1 cells, while IL-4 drives Th2 development.

IL-12 is a heterodimeric cytokine secreted by macrophages and dendritic cells, and can drive the differentiation of naive CD4^{+} T cells toward the Th1 cells with a cytokine profile of production of IFN-γ but not of IL-4 (Manetti et al., 1993; Heufler et al., 1996). Recent studies have shown that IL-12 has enormous potential as an immunomodulator and an adjuvant in therapeutic models of cancer, allergy and infectious diseases (Afonso et al., 1994; Trinchieri and Gerosa, 1996). However, several in vivo studies have demonstrated that recombinant IL-12 as an adjuvant may also increase IL-4 synthesis in antigen-primed CD4^{+} T cells in some situations (Bliss et al., 1996). The paradoxical increase in IL-4 synthesis driven by recombinant IL-12

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during *in vivo* immune responses may result from antigen nonspecific effects of IL-12, that may cause a rebound increase in the production of IL-10 (Meyaard *et al.*, 1996; Jeannin *et al.*, 1996; Gerosa *et al.*, 1996). De Smedt *et al.* (1997) reported that IL-10 induced the Th1/Th2 balance to Th2 *in vivo* by selectively blocking IL-12 systhesis by the antigen-presenting cells.

In this study, to minimize the antigen-nonspecific effects of IL-12 and to drive immune responses toward antigen-specific Th1 responses, we constructed an OVA/IL12 fusion protein that contained the T cell-dependent antigen, ovalbumin (OVA), covalently linked to murine IL-12. Our results showed that the OVA/IL12 protein enhanced OVA-specific IFN-γ production by lymph node CD4$^+$ T cells and promoted the production of anti-OVA IgG2a isotype antibody, while the simple mixture of free rIL-12 and OVA did not. These results suggest that the covalent linkage of the IL-12 molecule to the antigen would limit the effect of IL-12 to antigen-specific cells.

MATERIALS and METHODS

Mice

Seven- to nine-week old female BALB/c mice were purchased from the Jackson Laboratory (ME, USA).

Cell line and medium

Sf9 and High Five (*Trichoplasia ni*) insect cells were maintained in a T-75 flask as a monolayer, or in a culture flask as a suspension using EX-CELL 401 medium (JRH Biosciences, KS, USA) at 27°C. All cultures of lymph node cells from BALB/c mice were maintained in DMEM (Gibco BRL, NY, USA) supplemented with 10% fetal calf serum (Hyclone Laboratories, UT, USA), L-glutamine, 2- mercaptoethanol, and gentamycin.

Cytokines, antigens and antibodies

Murine recombinant IL-4, recombinant IFN-γ, and recombinant IL-12 were obtained from Genzyme Co. (MA, USA). Ovalbumin (OVA) was obtained from ICN Biomedicals (Montreal, PQ), and keyhole limpet hemocyanin (KLH) was purchased from the Calbiochem Co. (CA, USA). Anti-Ly 2.2 mAb (CD8) (HB 130) and anti-L3T4 mAb (CD4) (hybridoma GK1.5, ATCC TIB 207) were purified from ascitic fluids by ammonium sulfate precipitation. Anti-mIL-12p40 mAb C17.8 (rat IgG2a) were obtained from G. Trinchieri (Wistar Institute, PA, USA). Anti-OVA mAbs were produced by immunization of BALB/c mice with OVA in CFA, followed by injection with OVA in IFA in the footpads. The popliteal and axillary lymph node cells were harvested and fused with SP-2/0-Ag14 as previously described (Ozato and Sachs, 1981). Wells that demonstrated growth

were screened for binding to OVA by ELISA and were subcloned at 0.3 cells per well to assure clonality. Two anti-OVA mAbs, 6C1 (IgG1) and 3A11 (IgG2a), were prepared and used as standards for isotype-specific ELISA. Rabbit anti-OVA serum was purchased from Cappel Co. (Durham, NC, USA). 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). HRP-labelled goat anti-rabbit IgG was obtained from the Jackson Immunoresearch Laboratories Inc. (West Grove, PA) and HRP-labelled anti-rat IgG was purchased from Southern Biotechnology Associates Inc.

Construction of a baculoviral vector carrying an OVA/IL12 gene

A baculovirus vector encoding the OVA/IL12 gene for expression in insect cells was generated as follows. The gene for OVA was cloned by PCR from the OVA containing plasmid (pAcNeo-OVA), using primers containing the desired restriction sites. The p40 gene of IL-12 was inserted downstream of OVA gene separated by a spacer encoding Ser-Ser-Gly residues in a mammalian expression plasmid (Reff *et al.*, 1994) containing a SV40 origin of replication. The combined OVA/IL12p40 gene was then transferred into the baculovirus vector, pAcUW51 (Pharmigen Co., San Diego, CA), at the BamHI site downstream of the polyhedrin promoter. The p35 gene of IL-12, obtained from the plasmid pEDp35 (Kaufman *et al.*, 1991), was ligated into the same baculovirus vector (pAcUW51) at the EcoR1 site downstream of the p10 promoter.

Expression and purification of the OVA/IL12 fusion protein

The baculoviral vector carrying an OVA/IL12 gene was cotransfected with BaculoGold DNA into Sf9 insect cells (BaculoGoldTM system, PharminGen Co., USA). A single recombinant pAcUW51 plasmid containing the gene for the OVA/IL12 protein was isolated by plaque purification. The isolated recombinant baculovirus was amplified and then infected into High Five insect cells. One liter of baculovirus-infected High Five insect cell supernatant containing the OVA/IL12 protein was applied to an anti-OVA immunoaffinity chromatograpy prepared by coupling an anti-OVA mAb to CNBr-activated Sepharose 4B (Pharmacia Biotechnology Inc., Piscataway, NJ). The column was washed with 10 volumes of PBS, and the adsorbed OVA/IL12 protein was eluted with 50 mM glycine buffer containing 0.15 M NaCl (pH 3.0). Fractions were immediately neutralized in 1/20 volume of 1 M sodium

phosphate (pH 8.0), and fractions containing the protein were pooled and dialyzed against PBS. The purified OVA/IL12 protein were analyzed by SDS-PAGE, Western blot and ELISAs using mAb specific for OVA and for the IL-12p40 subunit.

SDS-PAGE and immunoblot analysis

SDS-PAGE and transfer of proteins to nitrocellulose by semidry electroblotting were performed as previously described (Schick et al., 1993). Blots were probed with either mouse anti-OVA serum at 1 ug/ml or rat anti-IL12p40 antibody (C17.8), washed and exposed to HRP-labelled anti-mouse IgG or HRP-labelled anti-rat IgG, respectively. The blots were then developed with the ECL system (Amersham, Arlington Height, IL), according to the manufacturer's directions.

In vitro stimulation of lymph node cells

BALB/c mice were injected in the footpads with the recombinant OVA or OVA/IL12 fusion protein (50 ul/footpad) as indicated in the figure legends. Seven days later, lymph node cells were obtained from the immunized mice and single cell suspensions were prepared as described (DeKruyff et al., 1995). The cells (5×10^5 cells/well) were distributed into each well of 96-well plate and incubated in vitro with varying concentrations of an antigen (OVA or KLH) for 3 days (proliferation assay) or 4 days (IFN-γ production assay).

Cytokine assays

The quantities of IFN-γ and IL-4 in culture supernatants of the lymph node cells were determined by a sandwich ELISA using mAbs specific for each cytokine (Genzyme Co., MA, USA). The biological activity of IL-12 in the OVA/IL12 protein was determined by the ability to stimulate the proliferation of phytohemagglutinin (PHA)-activated human lymphocytes as described previously (Coligan et al., 1991). Recombinant mouse IL-12 (Genzyme Co.) was used as a standard.

Determination of anti-OVA antibody isotypes

Mice were bled during the course of experiments, and the amount of OVA-specific antibody isotypes in the sera was measured by ELISA using mAbs specific for each isotype. Briefly, ELISA plates were coated with 100 ul of 5 ug/ml OVA per well. After coating, serial dilutions of sera were added to the plates and incubated overnight at 4°C. Anti-OVA IgG1 mAb (6C 1), anti-OVA IgG2a mAb (3A11), and anti-OVA total IgG were used as standards for quantitation of each IgG subclass. After washing, HRP-labelled anti-mouse IgG1, IgG2a, and IgG were added and incubated for 2 hrs at room temperature. After additional washing, o-phenylenediamine (OPD) substrate was added and de-

veloped for 10 min, and the OD was determined at 492 nm.

Proliferation assay

The lymph node cells (5×10^5 cells/well) were incubated in 96-well plates with varying concentrations of antigen (OVA or KLH) for 4 days in a total volume of 200 ul. The cells were pulsed with 1 uCi [^3H] TdR during the last 18 h of the culture. The cells were collected onto glass fiber filters with a PHD cell harvester (Cambridge Technology Inc., Watertown, MA), and the amount of radioactivity was measured in a β counter (Beckman Instruments, Fullerton, CA).

Statistics

The Student t test was used to determine the statistical differences between the various experimental and control groups.

RESULTS

The constructed OVA/IL12 fusion protein is biologically active

We generated an OVA/IL12 protein in a baculoviral expression system, as described in the Materials and Methods. The resulting protein consisted of ovalbumin covalently linked to the p40 subunit of IL-12 (IL12p40). The p35 subunit of IL-12 was linked to the IL12p40 subunit by a disulfide bond. A recombinant OVA protein was also produced in the baculovirus expression system and used as a control. The OVA/IL12 protein purified by anti-OVA immunoaffinity chromatography was analyzed by SDS-PAGE, and by Western imunoblotting using mAbs against OVA or the IL12p40 subunit (Fig. 1). Most of the purified protein contained both the p40 and p35 subunits of IL-12, and only a small fraction appeared to lack the p35 subunit. Biological activity of the OVA/IL12 protein was determined based on its ability to support proliferation of IL-12 dependent PHA-activated human lymphocytes. As shown in Fig. 2, the OVA/IL12 protein stimulated vigorous growth and proliferation of the PHA-activated human lymphocytes in a dose-dependent manner, while the recombinant OVA induced background proliferation. This stimulated proliferation of human lymphocytes by the OVA/IL12 protein was completely inhibited by a neutralizing antibody of IL-12, anti-p40 mAb (C17.8) (data not shown). The OVA/IL12 protein was about 50 fold less active than recombinant IL-12 on a mole for mole basis. The decreased activity of the OVA/IL12 protein may be partially due to the presence of an incomplete OVA/IL12p40 protein in the purified OVA/IL12 protein as shown in Fig. 1, or alternatively to steric hindrance of the adjacent OVA
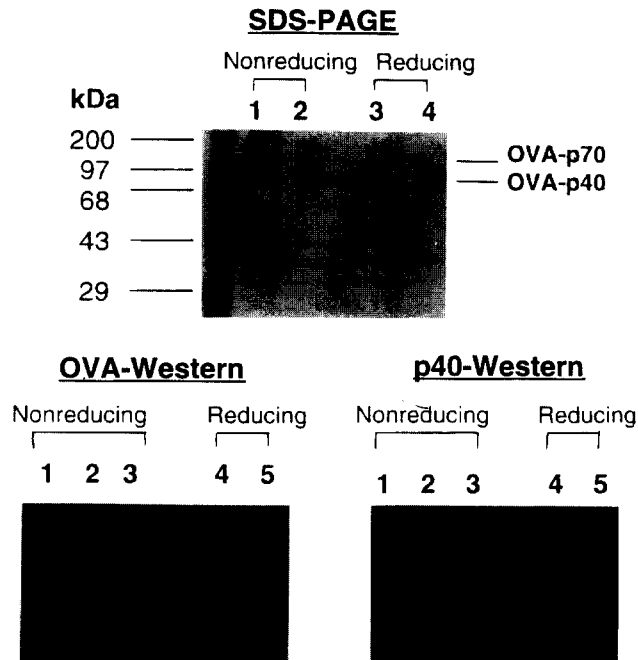
SDS-PAGE

Fig. 1. SDS-PAGE and Western blot analysis of the OVA/IL 12 fusion protein expressed in a baculovirus expression system. The recombinant OVA/IL12 protein and the recombinant OVA protein were subjected to SDS-PAGE under non-reducing and reducing conditions (SDS-PAGE). The proteins were electroblotted to nitrocellulose. Nitrocellulose strips were reacted with mouse anti-OVA mAb followed by HRP-labelled anti-mouse IgG (OVA-Western), or with rat anti-p40 mAb followed by HRP-labelled anti-rat IgG (p40-Western). SDS-PAGE and OVA-Western: lanes 2 and 4 (OVA/IL12), lanes 1 and 3 (OVA). p40-Western: lane 1 (rIL-12), lanes 3 and 5 (OVA/IL12), lanes 2 and 4 (OVA).

in the OVA/IL12 protein because the fusion protein contained only a three amino acid linker between the carboxy terminal of the OVA and the amino-terminal of the IL12p40. Decreased activity of cytokine fusion proteins has also been reported by Gillies et al. (1993) and Chen et al. (1994) with granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-4.

OVA-specific, Th1-dominated immune responses induced with the OVA/IL12 protein

To examine the immunogenicity of the OVA/IL12 protein, BALB/c mice were immunized with varying concentrations of the OVA/IL12 protein (0-100 pM) or the recombinant OVA (0-100 pM). Seven days later, the draining lymph nodes were removed and lymph node cells were stimulated in vitro with 100 ug of OVA or KLH. The proliferative response and IFN-γ produced by the responding cells were determined. Lymph node cells primed with the OVA/IL12 protein proliferated vigorously and produced high levels of IFN-γ in a dose-dependent manner of the OVA/IL12 protein, when stimulated in vitro with 100 ug of OVA (Fig. 3). In contrast, the lymph node cells did not proliferate or produce IFN-γ when the cells were in vitro
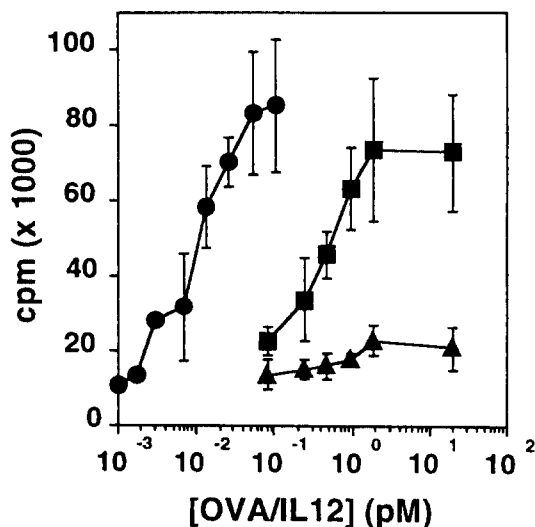

Fig. 2. Biological activity of the OVA/IL12 fusion protein. PHA-activated human lymphocytes were incubated with increasing concentrations of the OVA/IL12, recombinant OVA or recombinant IL-12. Proliferation was measured by ³H-thymidine uptake 16 hr later. The results are expressed as mean cpm±SD of triplicate determinations. ●—●: rIL-12, ■—■: OVA/IL12, ▲—▲: OVA.
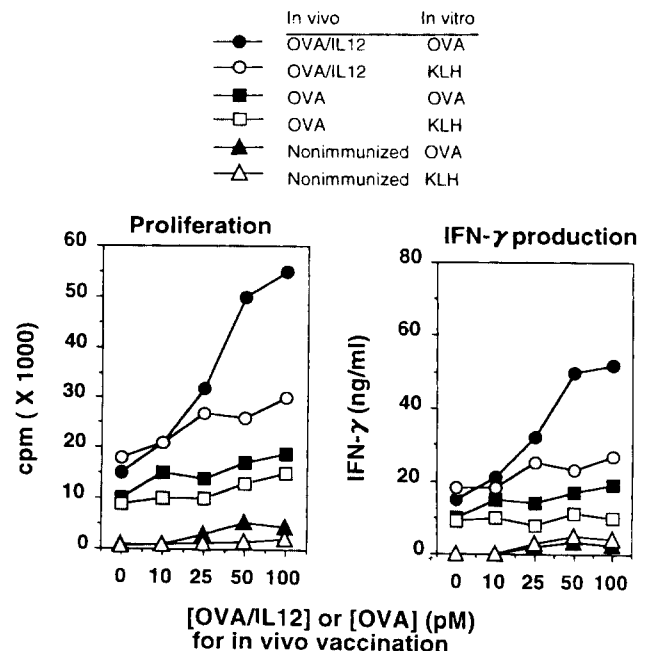

Fig. 3. OVA-specific proliferation and IFN-γ production induced with the OVA/IL12 protein. BALB/c mice were f.p. injected with increasing concentrations of the OVA/IL12 or the recombinant OVA. Seven days later, the lymph node cells were stimulated in vitro with 100 ug of OVA or KLH for 3 days and the proliferative response was determined. The lymph node cells were stimulated in vitro for 4 days, and the levels of IFN-γ in the supernatants were analyzed by ELISA. The values represent the mean of three separate experiments.
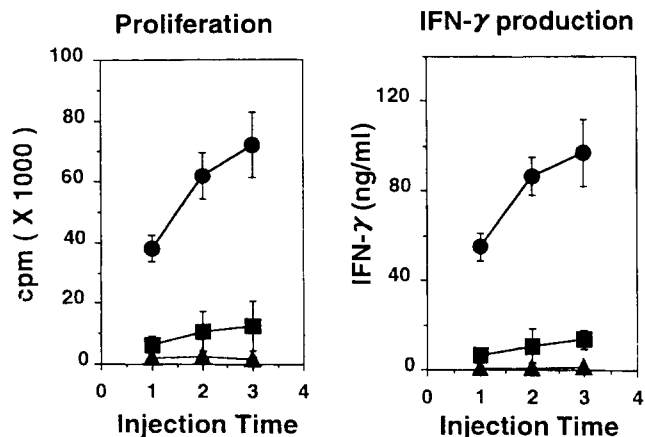
Fig. 4. Enhanced OVA-specific immune responses by multiple injections with the OVA/IL12 protein. BALB/c mice were f.p. injected with 50 pM OVA/IL12 protein at different times. Seven days after the injection, the proliferative response and IFN-γ production by OVA-specific cells were determined as described in the legend of Fig. 3. ●—●: OVA/IL12, ■- -■: OVA, ▲—▲: Nonimmunnized.

stimulated with KLH, an irrelevant antigen, indicating that the immune responses induced with the OVA/IL 12 protein were OVA-specific. The levels of IL-4 production in the immunized mice were relatively low
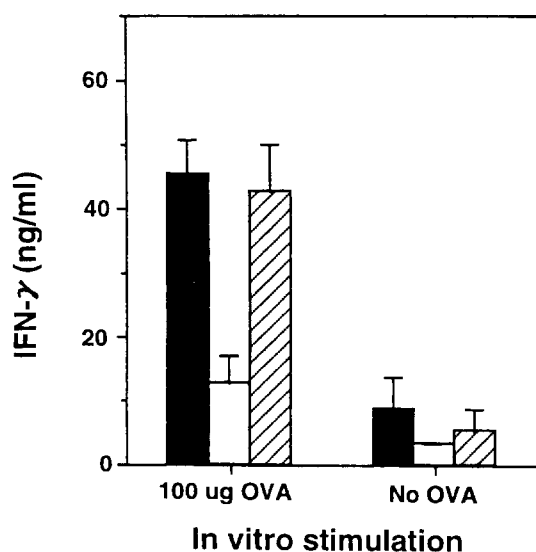

Fig. 5. CD4+ T cells are the major IFN-γ producing cell-type induced with the OVA/IL12 protein. BALB/c mice were twice f.p. immunized with 50 pM of the OVA/IL12 protein. Seven days later, the lymph node cells were incubated with anti-Ly2 (CD8) or anti-L3T4 (CD4) on ice for 10 min, followed by incubation with complement at 37°C for 45 min. After washing, the cells from each treatment group were stimulated with 100 ug OVA or with growth medium alone for 4 days and the IFN-γ production was determined by an ELISA. The data represent the mean±SD of triplicate determinations. ■: No depletion, □: CD4-depleted, ▨: CD8-depleted.
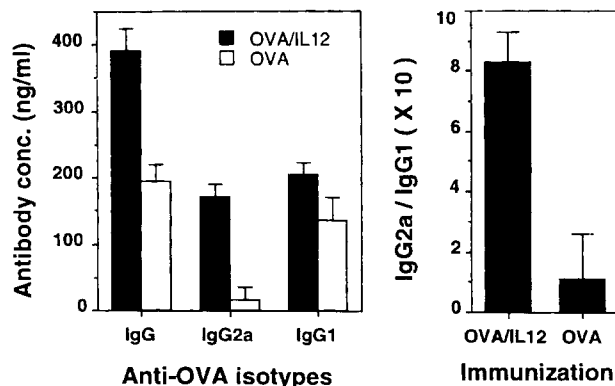

Fig. 6. Production of anti-OVA antibody isotypes in mice immunized with the OVA/IL12 protein. BALB/c mice were vaccinated i.p. with 50 pM of the OVA/IL12 or recombinant OVA (twice with a week interval). Four weeks after the injection, the levels of anti-OVA antibody isotypes in the sera were quantitated by isotype-specific ELISA. The values represent the mean±SD of five mice.

in all groups and not significantly different between groups (data not shown). The proliferative response and the IFN-γ production were dramatically increased by multiple injections with the OVA/IL12 protein (Fig. 4). In the cultures of cells from mice immunized with the OVA/IL12 protein, CD4+ T cells were the major producers of cytokines, since depletion of CD4+ T cells, but not CD8+, greatly reduced IFN-γ production (Fig. 5).

To examine the effect of the OVA/IL12 protein on the production of anti-OVA specific antibody isotypes, BALB/c mice were immunized twice i.p. with the OVA/IL12 (50 pM) or recombinant OVA (50 pM). Mice were bled 4 weeks after the injection, and antibody responses in the sera were determined by iso-type-specific ELISA. Immunization with the OVA/IL12 protein gave significantly higher titers of anti-OVA IgG and anti-OVA IgG2a than immunization with the OVA protein alone. Importantly, the OVA/IL12 protein induced the higher IgG2a/IgG1 ratios which were several folds higher than those induced with OVA alone (Fig. 6).

Covalent linkage between OVA and IL-12 limits the effect of IL-12 to OVA-specific cells

To investigate the importance of the physical linkage between OVA and IL-12 in the OVA/IL12 protein, mice were f.p. injected twice with the OVA/IL12 protein (50 pM) or a mixture of recombinant OVA (50 pM) and free rIL-12 (1 pM), or with the recombinant OVA protein (50 pM). The low amount of free rIL-12 (1 pM) was chosen because the biological activity of the OVA/IL12 fusion protein was 50 fold less active than an equivalent molar amount of free rIL-12. Cells from mice immunized with the OVA/IL12 protein pro-
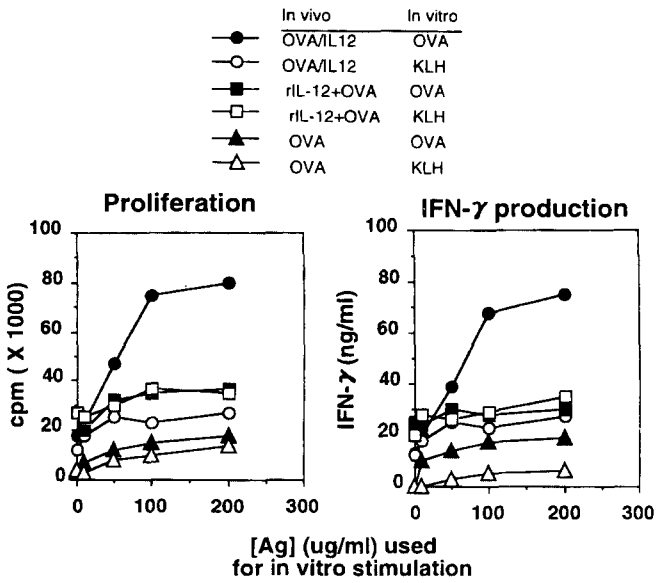
Fig. 7. OVA-nonspecific immune responses induced with a mixture of OVA and free rIL-12. BALB/c mice were immunized f.p. with the OVA/IL12, the recombinant OVA, or with a mixture of the recombinant OVA and free rIL-12 (twice with a week interval). Seven days after the immunization, the proliferative response and IFN-γ production were determined after *in vitro* stimulation with increasing amounts of OVA or KLH.

Fig. 8. The physical linkage between OVA and IL-12 is required for inducing an OVA-specific IFN-γ production. BALB/c mice were f.p. immunized with a mixture of the OVA/IL-12 protein and KLH, or with a mixture of free rIL-12, OVA and KLH (twice with a week interval). Seven days after the immunization, the lymph node cells were stimulated *in vitro* with increasing concentrations of OVA or KLH for 4 days, and the IFN-γ production was determined. The values represent the mean of three separate experiments.

liferated and produced IFN-γ when stimulated *in vitro* with OVA (Fig. 7). In contrast, cells from mice immunized with a mixture of OVA and free rIL-12 proliferated and produced IFN-γ independent of OVA, indicating that immunization with free rIL-12 resulted in the development of antigen-non specific cells that spontaneously proliferated and produced cytokines. Thus, physical linkage between the OVA and the IL-12 greatly enhanced antigen-specific effects of the IL-12.

To further investigate the importance of the physical linkage between the OVA and the IL-12, we mixed the OVA/IL12 protein with KLH, an irrelevant antigen, and injected with the mixture twice f.p. into mice. The lymph node cells from the immunized mice responded to OVA, not to KLH used for *in vitro* stimulation (Fig. 8). The lymph node cells from the immunized mice with a mixture of OVA, KLH and rIL-12 did not respond to both OVA and KLH. These results suggest that the IL-12 is required to be physically linked to the OVA protein for inducing an OVA-specific immune response.

DISCUSSION

In this report we have shown that the covalent linkage of IL-12 and the OVA confined the effect of IL-12 to OVA-specific cells, leading to a Th1-dominated immune response in an OVA-specific manner. The in-
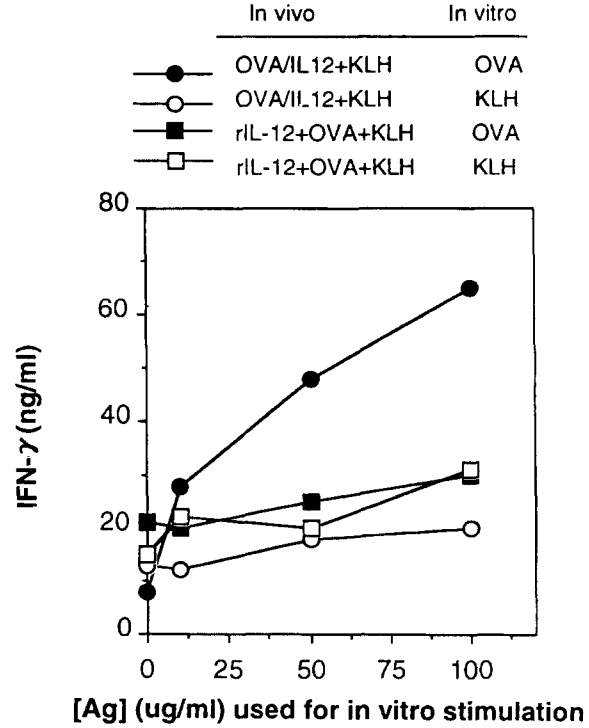
duced Th1 response was characterized by high titers of OVA-specific IgG2a antibody, and by high levels of IFN-γ production from OVA-specific CD4+ T cells. In contrast, free rIL-12 significantly increased antigen-nonspecific IFN-γ production, as seen by the *in vitro* IFN-γ production by lymph node cells in the absence of antigenic stimulation. Thus, the antigen non-specific effects of free rIL-12 were eliminated by the covalent linkage of IL-12 to the OVA protein. The OVA/IL12 protein also induced OVA-specific Th1-immune responses in C57BL/6 mice (H-2^h), indicating that these phenomena were not strain-specific (data not shown).

The mechanism by which the OVA/IL12 protein induces OVA-specific, Th1-dominated response compared with the OVA-nonspecific response of free rIL-12 is not clear. One possiblity is that the OVA/IL12 protein may efficiently induce OVA-specific, Th1 immune responses by increasing the *in vivo* half-life of IL-12 activity of the OVA/IL12 fusion protein compared with free rIL-12. 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. How-

ever, our co-immunization experiments demonstrate a much more absolute dependence on fusing the cytokine to the antigen for inducing antigen-specific immune responses. Immunization with a mixture of the OVA/IL12 protein and an irrelevant antigen, KLH, induced OVA-specific, not KLH-specific, Th1 immune responses. In addition, the simple mixture of KLH, OVA and free rIL-12 induced higher immune responses than immunization with KLH or OVA alone, which were not specific for both OVA and KLH. Therefore, the linkage between the OVA and the IL-12 allows the IL-12 activity to localize to sites in immune organs where OVA-specific cells are being activated. The presence of cytokines in the microenvironment of antigen-specifc immune responses gives advantages in T cell immunobiology and in cancer therapy, since direct injection of cytokine gene-containing vectors 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 OVA/IL-12 protein can convert the established Th2- to Th1-immune responses. Recently, ongoing Th 2 responses to *Leishmania major* were found to be converted to a healing Th1 response when BALB/c mice were treated during chronic infection with Pentostam (to reduce the parasite infection) together with IL-12 (Nabors *et al.*, 1995).

In conclusion, the OVA/IL-12 fusion protein can induce OVA-specific, Th1 dominated immune response, compared with OVA-nonspecific responses induced with the simple mixture of OVA and free rIL-12. These studies suggest that the covalent linkage of IL-12 to the OVA can reduce undesired antigen nonspecific immune responses and, therefore, antigen-IL-12 fusion protein may be beneficial in the treatment of diseases caused by undesired Th2 dominated responses, including allergic diseases and certain parasitic infections.

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REFERENCES CITED

Afonso, L.C., Scharton, T.M., Vieira, L.Q., Wysocka, M., Trinchieri, G. and Scott, P., The adjuvant effect of interleukin-12 in a vaccine against *Leishmania*

major. *Science*, 263, 235-237 (1994).

Bliss, J., Cleave, V.V., Murray, K., Wiencis, A., Ketchum, M., Maylor, R., Haire, T., Resmini, C., Abbas, A.K. and Wolf, S., IL-12, as an adjuvant, promotes a T helper 1 cell, but does not suppress a T helper 2 cell recall response. *J. Immunol.*, 156, 887-894 (1996).

Chen, T.T., Tao, M.H. and Levy, R., Idiotype-cytokine fusion proteins as cancer vaccines: relative efficacy of IL-2, IL-4, and granulocyte macrophage colony-stimulating factor. *J. Immunol.*, 153, 4775-4787 (1994).

Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M. and Strober, W., Current Protocols in Immunology, John Wiley and Sons, 1991.

DeKruyff, R.H., Fang, Y., Wolf, S.F. and Umetsu, D.T., IL-12 inhibits IL-4 synthesis in keyhole limpet hemocyanin-primed CD4+ T cells through an effect on antigen-presenting cells. *J. Immunol.*, 154, 2578-2587 (1995).

De Smedt, T., Van Mechelen, M., De Becker, G., Urbain, J., Leo, O and Moser, M., Effect of interleukin-10 on dendritic cell maturation and function. *Eur. J. Immunol.*, 27, 1229-1235 (1997).

Gerosa, F., Paganin, C., Peritt, D., Paiola, F., Scupoli, M.T., Aste-Amezaga, M., Frank, I. and Trinchieri, G., Interleukin-12 primes human CD4 and CD8 T cell clones for high production of both interferon-γ and interleukin-10. *J. Exp. Med.*, 183, 2559-2569 (1996).

Gilboa, E., Immunotherapy of cancer with genetically modified tumor vaccines. *Semin. Oncol.*, 23, 101-107 (1996).

Gillies, S.D., Young, D., Lo, K.M. and Roberts, S., Biological activity and *in vivo* clearance of antitumor antibody/cytokine fusion proteins. *Bioconj. Chem.*, 4, 230-235 (1993).

Heufler, C., Koch, F., Stanzl, U., Topar, G., Wysocka, M., Trinchieri, G., Enk, A., Steinman, R.M., Romani, N. and Schuler, G., Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. *Eur. J. Immunol.*, 26, 659-668 (1996).

Jeannin, P., Delneste, Y., Seveso, M., Life, P. and Bonnefoy, J.Y., IL-12 synergizes with IL-12 and other stimuli in inducing IL-10 production by human T cells. *J. Immunol.*, 156, 3159-3165 (1996).

Kaufman, R.J., Davies, M.V., Wasley, L.C. and Michnick, D., Improved vectors for stable expression of foreign genes in mammalian cells by use of the untranslated leader sequence from EMC virus. *Nucleic Acids Res.*, 19, 4485-4490 (1991).

Manetti, R., Gerosa, F., Giudizi, M.G., Biagiotti, R., Parronchi, P., Piccinni, M.P., Sampognaro, S., Maggi, E., Romagnani, S. and Trinchieri, G., Interleukin-12 induces stable priming for interferon gamma pro-

duction during differention of human T helper (Th) cells and transient IFN-γ production in established Th2 cell clones. *J. Exp. Med.*, 179, 1273-1283 (1993).

Meyaard, L., Hovenkamp, E., Otto, S,A. and Miedema, F., IL-12-induced IL-10 production by human T cells as a negative feedback for IL-12-induced immune responses. *J. Immunol.*, 156, 2776-2782 (1996).

Nabors, G.S., Afonso, L.C., Farrell, J.P. and Scott, P., Switch from a type 2 to a type 1 T helper cell response and cure of established Leishmania major infection in mice is induced by combined therapy with interleukin 12 and Pentostam. *Proc. Natl. Acad. Sci. USA*, 92, 3142-3146 (1995).

Ozato, K. and Sachs, D.H., Monoclonal antibodies to mouse MHC antigens. III. Hybridoma antibodies reacting to antigens of the H-2^b haplotype reveal genetic control of isotype expression. *J. Immunol.*, 126, 317-321 (1981).

Reff, M.E., Carner, K., Chambers, K.S., Chinn, P.S., Leonard, J.E., Raab, R., Newman, R.A., Hanna, N. and Anderson, D.R., Depletion of B cells *in vivo* by chimeric mouse human monoclonal antibody to CD20. *Blood*, 83, 435-445 (1994)

Schick, M.R., Ngujen, N.Q. and Levy, S., Anti-TAPA-1 antibodies induce protein tyrosine phosphorylation that is prevented by increasing intracellular thiol levels. *J. Immunol.*, 151, 1918-1925 (1993).

Trinchieri, G. and Gerosa, F., Immunoregulation by interleukin-12. *J. Leu. Biol.*, 59, 505-511 (1996).

Weinberg, A.D., English, M. and Swain, S.L., Distinct regulation of lymphokine production is found in fresh versus *in vitro* primed murine helper T cells. *J. Immunol.*, 144, 1800-1807 (1990).