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Dose-dependent Selective Priming of Th1 and Th2 Immune Responses Is Achieved Only by an Antigen with an Affinity over a Certain Threshold Level

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Helper $CD4^+$ T lymphocytes can be divided into two subsets, Th1 and Th2. The types of Th subsets activated during the adaptive immune response induction determine the efficacy of immune responses against the antigens introduced. Selective differentiation of subsets of $CD4^+$ T lymphocytes has been known to be influenced by several factors, such as the cytokine environment around the T cells, the specificity of antigen recognition by the T cell receptor, the expression of costimulatory molecules, and/or the dose of the antigen applied to stimulate the T cells. In this study, we tried to determine the influence of the antigen dose on the selective priming of T lymphocytes when an inefficient antigen was applied since all the conclusions drawn from previous experiments were based on experiments with immune systems which responded well against the antigens introduced. When the recombinant hen egg-white lysozyme (HEL) was used to stimulate immune responses in HEL low-responder C57BL/6 mice, dose-dependent selective priming of immune responses was not observed. However, when the variant antigen, which had been characterized as an efficient antigen in anti-HEL immune response induction in the low-responder mice, was applied, dose-dependent selective priming of Th immune responses was clearly demonstrated. These results suggested that dose-dependent selective priming of Th immune responses could be achieved only by the antigens with an affinity over a certain level.

Keywords: Dose; Epitope Peptide; T Cell Differentiation.

Introduction

The types of effector $CD4^+$ T lymphocyte activated during the adaptive immune response determine the efficacy of the immune response against the antigens introduced. Helper $CD4^+$ T (Th) lymphocytes can be divided into two distinct subsets, namely Th1 and Th2 cells (Arai *et al.*, 1997; Kim *et al.*, 1985; Mosmann *et al.*, 1986; Stout and Bottomly, 1989). Specific surface antigens which could be used to identify the subsets of $CD4^+$ T cells have not been characterized yet. Instead, the subsets of $CD4^+$ T cells are being identified on the basis of the cytokine profiles produced by them and their functional capabilities (Kim *et al.*, 1985). For instance, Th1-type $CD4^+$ T cells produce IL-2, IFN-γ, and TNF-β, and are responsible for the delayed-type hypersensitivity immune responses. On the other hand, Th2-type $CD4^+$ T cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, and are responsible for the B-cell-mediated humoral immune responses, especially for IgG1 and IgE responses, by inducing the B cells to secrete the antibodies (Cher and Mosmann, 1987; Coffman *et al.*, 1988; Killar *et al.*, 1987; Mossmann *et al.*, 1986; Stout and Bottomly, 1989). Although a third subset of $CD4^+$ T cells, Th0, has been described in a variety of priming conditions, the overlapping cytokine profiles associated with the population suggest that the subset may represent a mixed population of Th1 and Th2 subsets of $CD4^+$ T cells rather than a unique novel $CD4^+$ T cell population (Carding *et al.*, 1989; Openshaw *et al.*, 1995).

Both the Th1 and the Th2 subsets of $CD4^+$ T cells are derived from common precursor cells rather than

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Abbreviations: APL, altered peptide ligand; HEL, hen egg-white lysozyme; Th, helper T lymphocyte.

from two different pools of precursor Th cells. The selective differentiation of two subsets of $CD4^+$ T lymphocytes from common precursor cells is established during the initial priming of the cells and is influenced by the cytokine environment around the T cells (Hsieh *et al*., 1992; Seder and Paul, 1994; Seder *et al*., 1992). For example, IFN-γ and IL-12 are known to be the major cytokines involved in Th1 differentiation. IFN-γ exerts its function on the selective priming of Th1 cells by preventing the outgrowth of Th2 cells rather than by directly promoting the selective differentiation of Th1 cells. In contrast, IL-12 has no effect on the development of Th2 cells and is believed to prime Th1 cells directly. In contrast, the selective differentiation of Th2 cells is greatly influenced by the presence of IL-4 (LeGros *et al*., 1990; Swain *et al*., 1990). Although IL-10 has also been reported to promote the development of Th2 cells, the major effect of IL-10 on the selective differentiation of Th2 cells arises from the suppression of Th1 cell development rather than from the direct promotion of Th2 cell differentiation.

Besides the cytokine environment, the selective differentiation of subsets of $CD4^+$ T cells is known to be influenced by other factors, such as the specificity of antigen recognition by a T cell receptor, the expression of the costimulatory molecules, and the dose of the antigen applied to stimulate T lymphocytes (Constant and Bottomly, 1997). The influence of specificity of antigen recognition by a T cell receptor on the selective priming of $CD4^+$ T cells has been identified using altered peptide ligands (APLs). APLs are analogs of immunogenic peptide and have a modification on TCR contact sites of the epitope peptide (Sloan-Lancaster and Allen, 1996). APL could induce the unique pattern of T cell signal transduction, which was different from that induced by the cognate agonist peptide (Sloan-Lancaster *et al*., 1994). Owing to the ability of APLs to induce the unique pattern of signal transduction, APLs are believed to be able to promote the selective differentiation of $CD4^+$ T cells depending on the characteristics of the substituted amino acids within the APL. The selective differentiation of $CD4^+$ T cells is also known to be influenced by costimulatory molecules, such as CD28/CTLA-4 and B7-1/B7-2, which are expressed on antigen-presenting cells and T cells. For example, blocking of the CD28/B7 interaction greatly reduced the production of IL-2 and consequently the proliferation of Th1 cells, whereas Th2 cells were not affected (McKnight *et al*., 1994). Besides the report, evidence for the influence of costimulatory molecules on the selective priming of $CD4^+$ T cells has accumulated (Corry *et al*., 1994; King *et al*., 1995; Lu *et al*., 1994; Seder *et al*., 1994; Shahinian *et al*., 1993; Tao *et al*., 1997; Webb and Feldmann, 1995). Finally, the antigen dose used to stimulate immune responses could also have an influence on the selective priming of two subsets of $CD4^+$ T cells. The influence of the antigen dose on the selective priming of $CD4^+$ T cells was initially documented using bacterial flagellin as an antigen (Parish and Liew, 1972). In the study, high doses of immunogen tended to induce humoral immune responses, whereas low doses tended to induce cell-mediated immune responses. Interestingly, there are several reports where different doses of immunogen induced the opposite dichotomy of immune responses (Guery *et al*., 1996; HayGlass *et al*., 1986; Rogers and Croft, 2000; Wang *et al*., 1996). Currently, the influence of the antigen dose on the selective priming of $CD4^+$ T cells is believed to be dependent on the types of antigens used. For example, when a parasite was used as an antigen, a low dose of antigen induced Th1-like immune responses, whereas a low dose of soluble proteins tended to induce Th2-like immune responses (Bancroft *et al*., 1994; Bretscher *et al*., 1992; Sarzotti *et al*., 1996).

It should be noted regarding the conclusion drawn for the antigen dose dependency of the selective subset priming of $CD4^+$ T cells that all the results were obtained from studies using efficient antigens and that the animals responded well to the treated antigens. Because all the antigens introduced are not always well recognized by the immune system, we can assume that the dichotomy of dose-dependent selective priming of T cell differentiation with an inefficient antigen may be different from that with an efficient antigen. In this study, we analyzed the influence of the dose of an inefficient antigen on the selective priming of $CD4^+$ T cells using hen egg-white lysozyme (HEL) which was poorly recognized by HEL low-responder C57BL/6 mice. In addition, the results were compared with those from experiments using a variant HEL which was manipulated to be recognized efficiently by the same HEL low-responder C57BL/6 mice.

Materials and Methods

Chemicals, plastics, primers, and mice Unless otherwise specified, the chemicals and plastics used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively. Restriction enzymes and nucleic acid modifying enzymes were purchased from POSCO Chemical Co. (Sungnam, Korea). Oligonucleotide primers were purchased from Genotech (Yoosung, Korea). Four- to six-week old inbred C57BL/6 and C3H/He mice were purchased from the Korea Research Institute of Bioscience and Biotechnology (Yoosung, Korea).

Construction of plasmid vectors to produce wild-type and variant recombinant HEL proteins Complementary DNA for HEL was kindly provided by Dr. McCluskey (Brooks and McCluskey, 1993; Brooks *et al*., 1991). To construct the plasmid vector for the production of recombinant HEL (rHEL), which did not contain the leader sequence of the HEL, PCR

amplification was carried out and the PCR product was cloned into *E. coli* expression vector pQE30 (Fig. 1). Sequences of forward primer with the site for *Bam*H I and reverse primer with the site for *Hin*d III were 5′-CAC GGA TCC AAA GTC TTT GGA CGA TGT-3′ and 5′-ATT AAG CTT TCA CAG CCG GCA GCC TCT GAT-3′, respectively.

To generate the gene construct for rHELR61A which included alanine instead of arginine at HEL 61, sequential PCR-based *in vitro* mutagenesis was performed (Higuchi *et al.*, 1988; Ho *et al.*, 1989; Kadowaki *et al.*, 1989). The initial PCR reaction was carried out in two separate tubes. In the first tube, the forward primer which had been used to amplify the rHEL gene and a middle reverse primer which contained the codon for mutation (5′-CGT TGC ACC ACC AGG CGC TGT TGA TCT GTA-3′), were applied to amplify the partial rHEL gene which represented the 5′ part of rHEL with the mutated codon for HEL 61. Similarly, a middle forward primer which contained the codon for mutation (5′-ACA GAT CAA CAG CGC CTG GTG GTG CAA CGA-3′) and the reverse primer which had been used to amplify the rHEL gene were applied to amplify the partial rHEL gene which represented the 3′ part of rHEL with the mutated codon for HEL 61. After the initial PCR reaction, PCR products were eluted and mixed together in one tube and the second PCR reaction was performed for ten cycles without addition of primers. Finally, the third PCR reaction was performed with the forward and reverse primers which had been used to amplify the rHEL gene, and the PCR product was cloned into pQE30 vector. The mutated codon for HEL 61 was confirmed through nucleotide sequencing.

Expression and purification of recombinant HEL antigens Wild-type and mutant rHEL were produced in *E. coli* using the constructed pQE30-rHEL and pQE30-rHELR61A vectors, respectively, as recommended by the manufacturer (Qiagen, Chatsworth, CA, USA). The recombinant proteins were purified using a Ni^{+}-NTA agarose column and their molecular weights were confirmed through SDS–PAGE (Fig. 2A). The antigenicity of the recombinant proteins was also confirmed through Western blot analysis by using monoclonal anti-HEL antibody produced in this laboratory (Fig. 2B).

Immunization For the immunization, five mice per group were initially injected subcutaneously with 6 and 60 μg of antigens emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA) for low- and intermediate-/high-dose immunization, respectively. For the analysis of

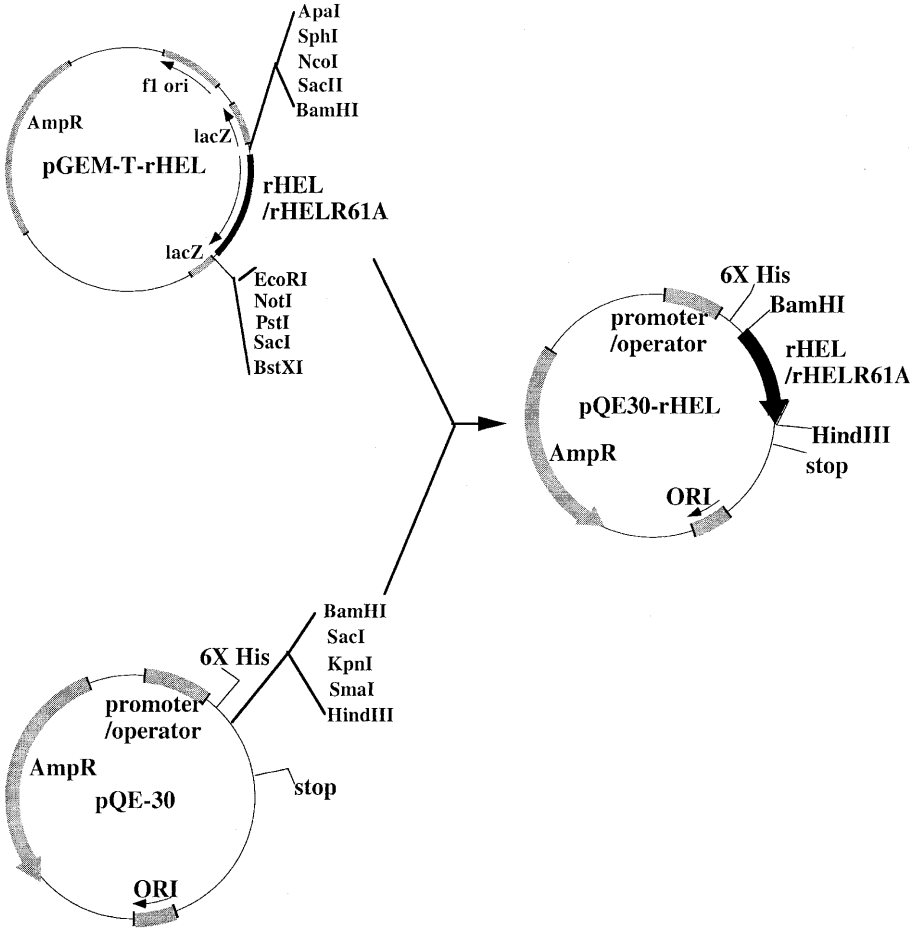

Fig. 1. Construction of pQE30-rHEL and pQE30-rHELR61A plasmid vectors, which contain cDNAs for HEL without a signal peptide sequence and for HEL with alanine at HEL 61 instead of arginine, respectively, for the expression of recombinant protein antigens in *E. coli*. The arrows indicate the direction of transcription and the closed bars represent the coding genes.

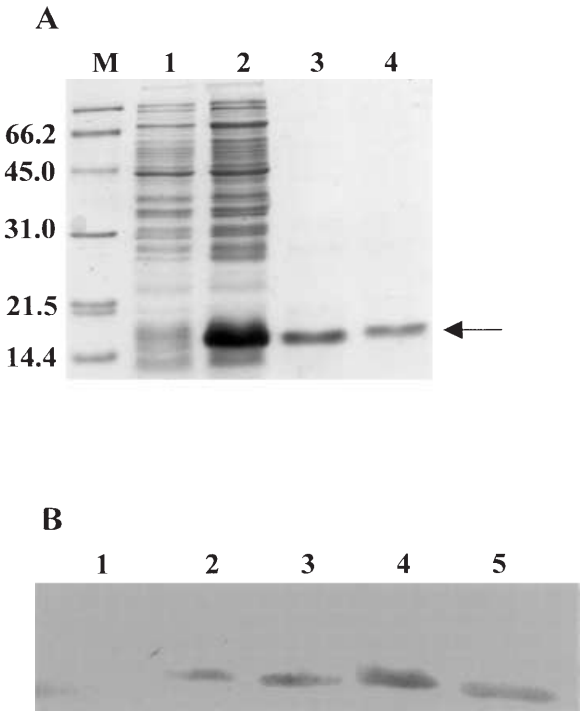

Fig. 2. Production and purification of rHEL and rHELR61A. **A.** The proteins were analyzed through 12% SDS–PAGE. Lanes M, 1, 2, 3, and 4 represent the molecular size markers, uninduced cell lysate, induced cell lysate, purified rHEL, and purified rHELR61A, respectively. The numbers on the left represent the molecular mass of the protein marker in kilodaltons. An arrow indicates the expected size of the rHEL proteins. **B.** Western blot analyses of the recombinant HEL proteins using anti-HEL antibody. Lanes 1, 2, 3, 4, and 5 represent results obtained from uninduced cell lysate, induced cell lysate for rHEL, purified rHEL, induced cell lysate for rHELR61A, and purified rHELR61A, respectively.

antibody immune responses, the same mice were boosted subcutaneously 2 weeks after the first injection with the same dose of antigen emulsified in incomplete Freund's adjuvant (Difco Laboratories). For the analysis of T cell proliferation, only single immunization was performed without a booster immunization.

Measuring the level of antibody immune responses The level of specific antibody immune responses was measured through ELISA, with the sera collected from the blood drawn 5 d after the booster immunization. For the ELISA, each well of the microtiter plate (Nunc, Denmark) was coated overnight at 4°C with 500 ng of HEL in 50 μl of 0.05 M carbonate–bicarbonate coating buffer (pH 9.6). After washing three times with PBS, the wells were blocked for 1 h at 37°C with blocking solution (1% skimmed milk and 0.02% Tween-20 in PBS). After the blocking solution had been washed out, the sera were serially diluted twofold with blocking solution and added into each well. After 2 h incubation at room temperature, the wells were washed and secondary antibodies were added. For the determination of the titer of total anti-HEL antibodies, alkaline phosphatase conjugated goat anti-mouse Ig (Promega, Madison, WI, USA) was used as the secondary antibody. For the determination of antibody titers of each isotype, the secondary antibody included in the monoclonal antibody-based mouse immunoglobulin isotyping kit (Pharmingen, San Diego, CA, USA) was used. Finally, the color was developed by adding disodium *p*-nitrophenyl phosphate substrate into each well and the optical density was measured at 405 nm using a SpectraCount^{TM} (Packard Instrument Co., Downers Grove, IL, USA) ELISA reader.

Lymph node cell proliferation Nine-to-ten days after immunization, draining lymph nodes were collected from each of five mice per group and pooled together to prepare single cell suspensions. The cells were cultured in triplicate in flat-bottomed 96-well microtiter plates at 5×10^5 cells per well in the presence of various concentrations of antigen and 0.5% normal syngeneic sera. Proliferative responses were measured by the addition of 1 μCi of [*methyl*-^{3}H]TdR (Amersham Pharmacia Biotech, Piscataway, NJ, USA) per well for the last 16–18 h during a 96 h culture period. The cells were harvested using a 96-well plate harvester (Inotech, Switzerland) and the incorporated tritium content was determined using a liquid scintillation counter (Packard Instrument Co.).

Cytokine ELISA ELISA to determine the level of cytokine expression by the antigen-stimulated T cells was provided by the Bank for Cytokine Research (Chonbuk National University, Chonju, Korea). Briefly, lymph node cells were collected from the immunized mice 9–10 d after the initial injection of antigen and the cells were cultured with antigen at 5×10^6 cells/ml in 24-well microtiter plates for 48 h. The levels of IL-4 and IFN-γ in the culture supernatants were determined using the cytokine-specific ELISA kit (Endogene, Cambridge, MA). The concentrations were determined on the basis of standard curves generated using known concentrations of recombinant proteins.

Statistical analyses The statistical significance of the data was analyzed through an unpaired two-tailed *t*-test using SigmaPlot® software.

Results

Production of recombinant HEL antigen In this study, the pQE vector-based *E. coli* expression system was used to produce the recombinant HEL proteins. The rHEL and rHELR61A produced were confirmed for their molecular weight through SDS–PAGE (Fig. 2A) and for their antigenicity through Western blot analysis (Fig. 2B) using HEL-specific monoclonal antibody. However, since the recombinant proteins produced through the system always contained 6× histidine at their N-termini, there was a possibility that the MHC-restricted pattern of immunogenicity of rHEL might be different from that of native HEL. To exclude this possibility, we tested if the immunogenicity of rHEL was similarly low as native HEL in HEL low-responder C57BL/6 mice. As shown in Fig. 3, the pattern of the

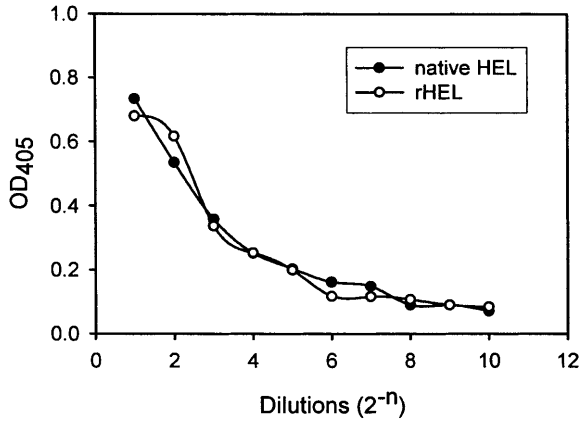

Fig. 3. Level of anti-HEL antibody immune responses induced by an injection of rHEL and native HEL in HEL low-responder C57BL/6 mice. The open and closed circles represent the results obtained from rHEL and native HEL injected mice, respectively. The results represent the mean values of duplicates. The same experiments were repeated five times and the figure shows a representative result.

antibody immune response induced by rHEL was almost the same as that induced by commercially available native HEL which was purified from hen egg-white. In addition, the levels of antibody titers induced in C57BL/6 mice by both native HEL and rHEL were relatively very low compared to that in HEL high-responder C3H/He mice (data not shown). These results confirmed that the MHC-restricted pattern of the immune response induced by rHEL was similar to that induced by native HEL and that the MHC-restricted pattern of immune response induction against HEL was well conserved in the mice used in this study.

Dose-dependent immune response induction by an inefficient antigen We initially tested whether there was any dose-dependent change in the anti-HEL antibody immune response induction by an inefficient antigen (Fig. 4A). As shown in Fig. 4A, the level of the anti-HEL antibody responses induced by a low dose (6 μg) of rHEL, which is known as an inefficient antigen in the mouse strain tested, was relatively very low. Similarly, even the injection of an intermediate dose (60 μg) of rHEL also induced a very low level of anti-HEL antibody immune responses. These results suggested that the antigen dose did not have any influence on the induction of antibody immune responses when an inefficient antigen was used.

In the lymph node cell proliferation assay, a low-dose injection of the inefficient rHEL antigen did not result in any detectable stimulation of HEL-specific T cells (Fig. 4B). Similar to the results for antibody immune responses, even the intermediate dose of rHEL did not change the low level of HEL-specific T cell stimulation. The levels observed were almost the same as that of the

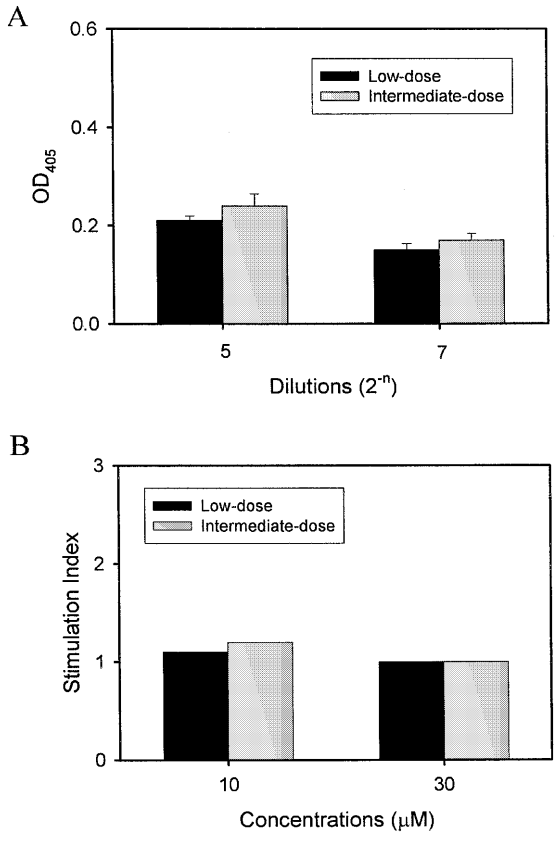

Fig. 4. Levels of (**A**) anti-HEL antibody and (**B**) anti-HEL T cell proliferative responses induced by the injection of rHEL as an antigen. The results of anti-HEL antibody and T cell proliferative responses represent the mean values of duplicates and triplicates, respectively. The same experiments were repeated three times and the figure shows a representative result. Stimulation indices were obtained by dividing the cpm of test samples by that of negative control. The cpms for negative control of low and intermediate doses of antigen injected experiments were 1,816 ± 46 and 442 ± 133, respectively.

background, since the stimulation indices were around 1.0. These results suggested that the antigen dose did not have any influence on the HEL-specific T cell stimulation by the injection of inefficient antigen as well. Collectively, different antigen doses did not have any influence on the selective priming of the immune response induction when the inefficient antigen was applied.

Dose-dependent immune response induction by an efficient antigen In order to see if there was any relationship between the efficiency of the antigen and the dose-dependent skewing of the immune response, we checked the influence of the antigen dose on immune response induction using a more efficient antigen than rHEL. An rHEL variant, rHELR61A, was a mutant rHEL, of which the arginine at HEL 61 had been substituted with alanine, and it was confirmed that injection of

rHELR61A induced very efficient anti-HEL immune responses compared to rHEL. When rHELR61A was used to immunize the HEL low-responder C57BL/6 mice at a low dose, the anti-HEL antibody response was much higher than that induced by rHEL (Figs. 4A and 5A). An intermediate dose of rHELR61A induced more efficient anti-HEL antibody immune responses than an injection of a low dose of antigen and the level of the anti-HEL antibody immune response induced by rHELR61A was about twice as high as that induced by rHEL (Figs. 4A and 5A).

The dose-dependent difference of immune response induction was clearly represented when rHELR61A was used to stimulate HEL-specific T cells (Fig. 5B). As shown in the figure, a low-dose injection of rHELR61A induced a relatively low level of HEL-specific T cell stimulation. However, the injection of an intermediate dose of rHELR61A induced a very efficient stimulation of HEL-specific T cells. These results suggested that the dose-dependent selective immune response induction by an antigen could be obtained only with the efficient antigen.

Characterization of the dose-dependent immune response induction by an efficient antigen In order to analyze the characteristics of the immune responses induced by rHELR61A, the levels of the key cytokine molecules involved in the selective priming of Th1 and Th2 immune responses were determined after T cell stimulation (Fig. 6). As shown in the figure, the level of IFN-γ, a key cytokine molecule for the selective differentiation of

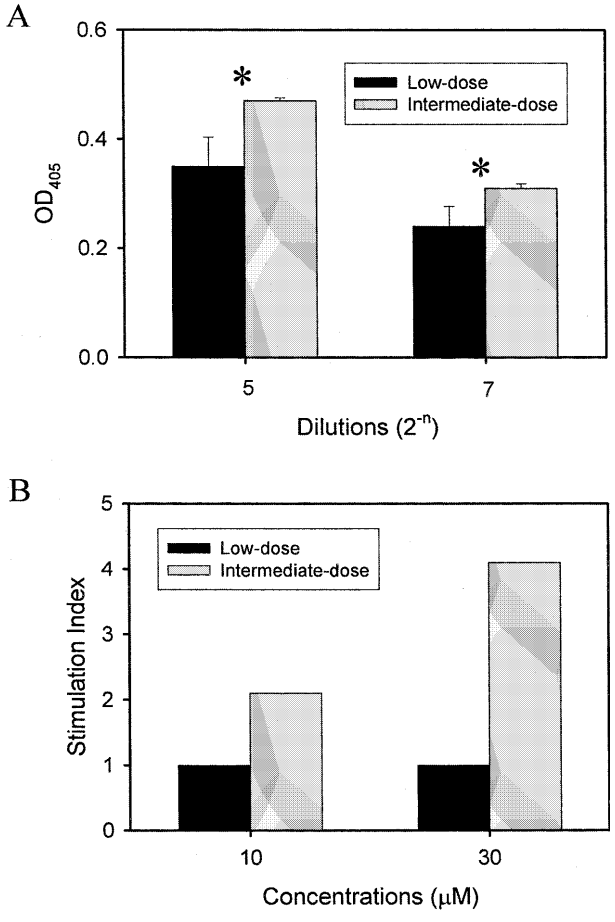

Fig. 5. Levels of (**A**) anti-HEL antibody and (**B**) anti-HEL T cell proliferative responses induced by the injection of rHELR61A as an antigen. The results of anti-HEL antibody and T cell proliferative responses represent the mean values of duplicates and triplicates, respectively. The same experiments were repeated three times and the figure shows a representative result. Stimulation indices were obtained by dividing the cpm of test samples by that of negative a control. * P values were less than 0.05. The cpms for negative control of low and intermediate doses of antigen-injected experiments were 4,976 ± 62 and 3,745 ± 48, respectively.

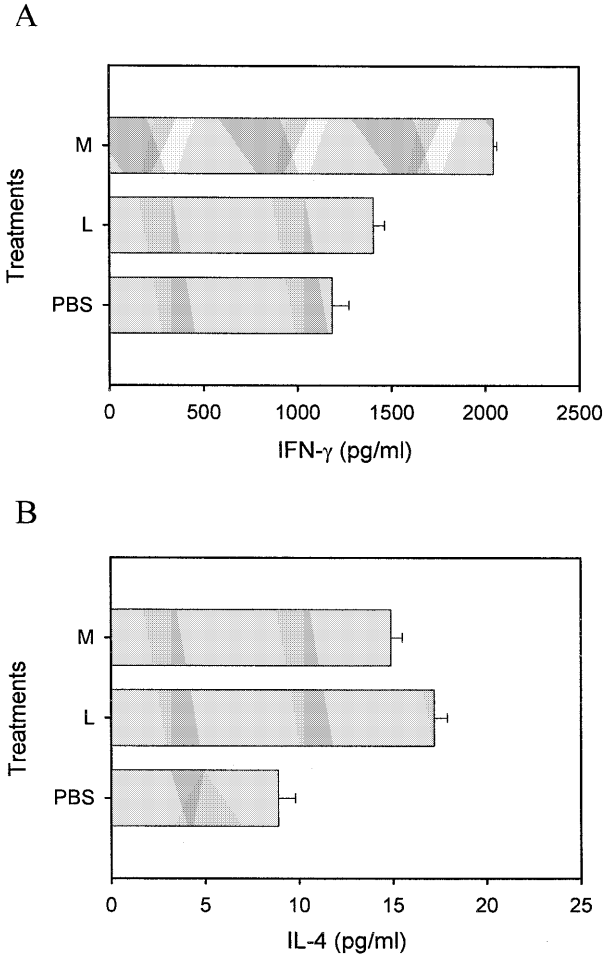

Fig. 6. Levels of (**A**) IFN-γ and (**B**) IL-4 production after the stimulation of the lymph node cells with native HEL. PBS, L, and M represent the results obtained using lymph node cells collected from mice injected with PBS, a low dose of antigen, and an intermediate dose of antigen, respectively. This result represents the mean values of duplicates. The same experiments were repeated three times and the figure shows a representative result.

Th1 cells, produced by the stimulation of T cells from the mice injected with an intermediate dose of antigen was higher than that with a low dose of antigen. In contrast, the level of IL-4, a key cytokine molecule for the selective differentiation of Th2 cells, produced by the stimulation of T cells from the mice injected with an intermediate dose of antigen was lower than that with a low dose of antigen. These results suggested that when the efficient antigen was used, dose-dependent selective priming of Th1 and Th2 cells was observed: a low dose antigen induced preferentially the expression of IL-4, while an intermediate dose of antigen induced the preferential expression of IFN-γ.

To confirm the dose-dependent selective priming of $CD4^+$ T cells induced by rHELR61A, isotypes of the anti-HEL antibodies which were induced by the injection of a low or an intermediate dose of rHELR61A were determined (Fig. 7). As shown in the figure, IgG1 production induced by the injection of a low dose of rHELR61A was more efficient than that induced by an intermediate dose of the same antigen. However, in the case of induction of IgG2a and IgG2b, an intermediate dose of rHELR61A was more efficient in antibody induction than a low dose of the same antigen. These results confirmed the observations that the injection of low and intermediate doses of rHELR61A induced the skewed production of Th2- and Th1-type cytokines, respectively.

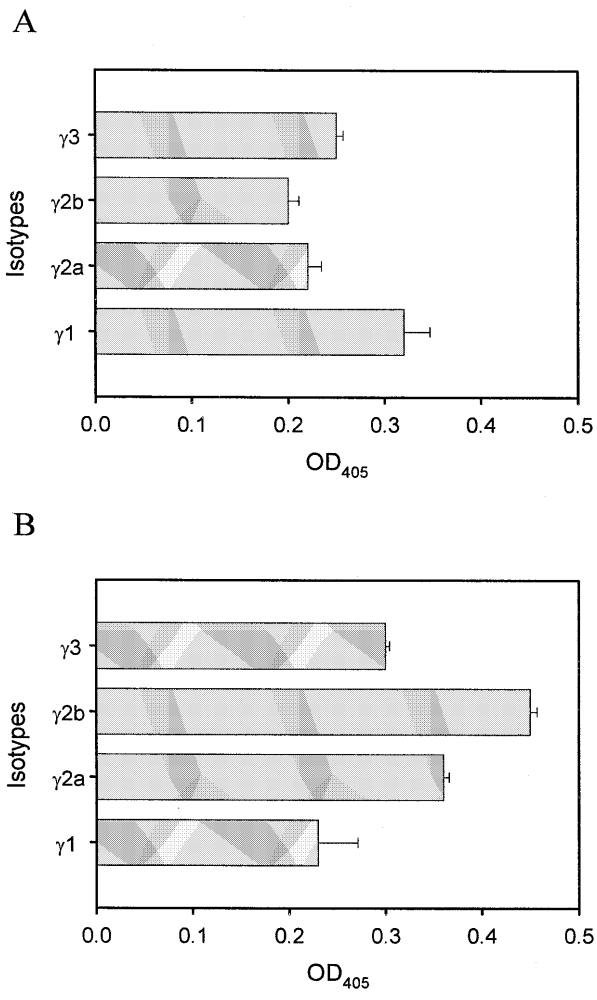

Fig. 7. Level of antibodies with each isotype determined using sera drawn from mice injected with (**A**) a low dose of antigen and (**B**) an intermediate dose of antigen, respectively. γ1, γ2a, γ2b, and γ3 represent the isotypes of IgG1, IgG2a, IgG2b, and IgG3, respectively. This result represents the mean values of duplicates. The same experiments were repeated three times and the figure shows a representative result.

Discussion

HEL is a model antigen whose biochemical characteristics and haplotype-dependent restriction of immune responses were well analyzed (Schwartz, 1986). For example, *k*- or *a*-haplotype bearing mice are known as high responders against the HEL antigen, whereas *b*- or *d*-haplotype bearing mice are known as low/nonresponders. In addition, recognition patterns of HEL epitopes were well characterized in several different strains of mice (Gammon *et al.*, 1987; 1991). Among the T cell epitopes identified, a lot of interest has been concentrated on HEL 46-61, since this region was a prominent epitope recognized by HEL high-responder C3H/He mice, whereas T cells specific to the same epitope were hardly induced in HEL-injected low-responder C57BL/6 mice. Consequently, the region has been considered as a good model system to study the difference in immune recognition between high and low responders against the same antigen. Previously, the arginine at HEL 61 was identified as an inhibitory amino acid residue for the binding of HEL 46-61 epitope peptide onto the MHC class II molecules of low-responder mice owing to the large size of the side chain of the amino acid (Jang *et al.*, 1994; Mikszta *et al.*, 1997). Consequently, substitution of the arginine with either alanine or serine, which is an amino acid with a short side chain, enhanced the immunogenicity of HEL in HEL low-responder mice. In this regard, HEL and rHELR61A are the good model antigen system to compare the pattern of immune response induction between inefficient and efficient antigens in low responders. In this study, the recombinant protein antigens were prepared using the pQE-based *E. coli* expression system, in which the recombinant proteins contained a 6× histidine tag at their N-termini. Owing to the extra amino acids, there could be an argument that the pattern of immune responses induced by rHEL might be different from that of native HEL. This argument could be excluded by the result that the pattern of anti-HEL antibody immune responses with rHEL was completely matched with that of native HEL (Fig. 3).

The results of previous studies on antigen dose-dependent selective priming of Th cells were obtained from immune systems which responded well against the injected antigens (Constant *et al*., 1995; Guery *et al*., 1996; Morokata *et al*., 2000; Wang *et al*., 1996). In this study, we compared the influence of antigen dose on selective priming of Th1 and Th2 types of immune responses between the inefficient and efficient antigens in low responders. The influence of the antigen dose on the selective induction of Th1 and Th2 types of immune responses was not observed in antibody and T cell proliferation responses when the inefficient rHEL was injected (Fig. 4). However, when the efficient antigen, rHELR61A, was used, dose-dependent selective priming of the Th1 and Th2 immune responses was clearly observed. For example, a low-dose injection of rHEL-R61A induced efficient Th2 immune responses as reported previously using soluble antigens (Guery *et al*., 1996). This was concluded from the fact that IL-4, a key cytokine for the differentiation of Th2-type $CD4^+$ T cells, was efficiently produced by the antigen-stimulated T cells isolated from the mice injected with a low dose of rHELR61A (Fig. 6). In addition, the result that a dominant isotype of anti-HEL antibodies in the mice injected with a low dose of rHELR61A was IgG1 suggested efficient isotype switching from IgM to IgG1, which could be mediated by IL-4 (Fig. 7). Similarly, an intermediate dose of antigen induced more efficient Th1 immune responses than a low dose of antigen as reported previously using soluble antigens (Hosken *et al*., 1995), which was concluded from the fact that IFN-γ, a key indicator cytokine for the differentiation of Th1-type $CD4^+$ T cells, was produced efficiently in mice injected with an intermediate dose of rHELR61A (Fig. 6). Also, the result that dominant isotypes of anti-HEL antibodies in the mice injected with an intermediate dose of rHELR61A were IgG2a and IgG2b suggested efficient isotype switching from IgM to both IgG2a and IgG2b, which might be mediated by IFN-γ (Fig. 7). These results collectively confirmed that dose-dependent selective priming of Th1 and Th2 immune responses could be obtained only with an efficient antigen.

The dose-dependent priming of Th1 and Th2 immune response induction did not seem to be clearly illustrated in the antibody immune response induced by rHELR61A (Fig. 5). We believe that this was due to the combined effect of Th cell stimulation: Th1 cells induced the expression of IFN-γ and consequently induced the isotype-switching from IgM to IgG2a and IgG2b, while Th2 cells induced the production of IL-4 and consequently induced the isotype-switching from IgM to IgG1. Therefore, even though there was selective priming of Th1 and Th2 cells depending on the antigen dose, the effect of the selective priming could not be clearly demonstrated in the total antibody titers (Fig. 7).

Cytokine profiles of the T cells from the rHELR61A-injected mice showed that the dose-dependent expression of the cytokine profile was clearly demonstrated in IFN-γ, although the profile of IL-4 was not clearly demonstrated (Fig. 6). We assume that this was due to the difference in the working pattern of IFN-γ and IL-4 such that IFN-γ was involved in the selective priming of Th1 cells by suppressing the priming of the Th2 cells rather than by directly priming the target cells, whereas IL-4 primed the Th2 cells directly (Le Gros *et al*., 1990; Swain *et al*., 1990). Therefore, the difference in the level of IFN-γ was more dramatic than that of IL-4.

The dose-dependent selective priming of Th cells which had been shown only by the efficient rHELR61A antigen was believed to be mainly due to the increased affinity of epitope peptide over a certain threshold level. There could be an argument that the observed dose-dependent selective priming of Th immune responses with an efficient antigen simply represented the effect of the increased density of the epitope peptides but not the effect of increased affinity of the epitope peptides. This argument could be ruled out by two aspects of HEL antigen recognition by T lymphocytes. First, HEL 46-61 is not a major HEL epitope recognized by T cells from HEL low-responder C57BL/6 mice. Instead, HEL 13-35 and HEL 74-90 are known as dominant T cell epitopes recognized by HEL-injected C57BL/6 mice, although the peptides are still very inefficient in T cell stimulation (Gammon *et al*., 1987). Therefore, if the antigen dose-dependent selective priming of Th immune responses simply represented the increased density of the epitope peptide, an intermediate and a high dose injection of inefficient rHEL should also induce the selective priming of Th2 immune responses by the increased density of the major T cell epitopes. However, we could not detect that kind of selective priming of Th2 immune responses by intermediate- and high-dose injection of rHEL (Fig. 4 and data not shown). Second, binding of epitope peptide onto MHC class II molecules is a prerequisite of T cell stimulation. This means that even if the density of a certain epitope peptide is very high, the critical step for the epitope peptide to stimulate the T cells is its binding onto the MHC class II molecule. Therefore, we believe that the selective priming of Th2 immune responses obtained by rHELR61A not by rHEL was due to the increased affinity of the epitope peptide produced from the modified antigen. Although we could not completely exclude the possibility that the epitope density was increased by the epitope modification, we could define that the principal reason for the selective priming of Th immune responses was obtained by the increased affinity of the peptide which was evolved from the epitope modification. The report that peptide analogs with different affinities for MHC class II molecules altered the cytokine profile of T helper cells supports the concept that the affinity of the antigenic

epitope was one of the critical factors involved in the selective priming of $CD4^+$ T cell immune responses (Chaturvedi *et al.*, 1996). In addition, our previous result that the epitope peptide with alanine at HEL 61 was higher than that with arginine in its binding affinity onto I-A^b also supported the conclusion (Jang *et al.*, 1994). We are currently comparing the production of epitope peptides around the HEL 46-61 region between rHEL and rHELR61A to narrow down the ultimate processed products to clearly understand the reason for the increased affinity of the epitope peptide produced from the modified antigen.

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