3.7. Detection of MPT51-specific $CD8^+$ T cells using MHC class I/peptide tetramer

Since we had identified both the $CD8^+$ T cell epitope of MPT51 and its class I restriction molecule, it became possible to detect MPT51-specific $CD8^+$ T cells using MHC class I/peptide tetramer. Three-color flow cytometric analysis of MPT51-immune splenocytes employing the tetramer (PE-conjugated H2-D^d/MPT51 24–32 [GGPHAVYLL]), and CyChrome-conjugated anti-CD4 and FITC-conjugated anti-CD8 mAbs revealed that 3.7% of $CD8^+$ T cells was specific for MPT51 (Fig. 7). As expected, no cells were stained by the tetramer in the $CD4^+$ T cell population in MPT51-immune mice or in naïve splenocytes.

4. Discussion

In this study, we have used a molecular approach for the identification of $CD8^+$ T cell epitopes in the MPT51 protein of *M. tuberculosis*, a novel secreted antigen that was previously shown to be protective in vaccinated mice (Miki et al., 2004). Employing the retroviral expression system, we successfully identified one $CD8^+$ T cell epitope, MPT51 24–32 (GGPHAVYLL), in BALB/c mice.

Progressing from longer expressed fragments (120–140 aa in length) of MPT51 to shorter peptides, we were able to narrow down the MPT51 epitope within the first N-terminal 60 amino acids. Attempts to use constructs that expressed this region in 20-mer stretches did not reveal the epitope, a problem that was resolved by creating fusion constructs of these peptides with ubiquitin. One possible reason is that the 20-mer products are poorly ubiquitinated by ubiquitin ligase. Our observation that cotranslational ubiquitination of the 20-mer fragment overcame the problem supports this possibility. Cotranslational ubuquitination assists the polyubiquitination of the 20-mer, which has been shown to be required for recognition by the proteasome (Thrower et al., 2000).

Ubiquitination is a pivotal step in cytosolic protein degradation and MHC class I antigen presentation. Proteins destined for degradation by the 26S proteasome are commonly modified by a multi-ubiquitin chain anchored to an internal ϵ-NH_2 group by one or more lysine residues. There are several proteins that are ubiquitinated at the amino-terminal α-NH_2 group rather than at an internal lysine residue for its degradation (Hou et al., 1994; Treier et al., 1994; Ikeda et al., 2002). In the case of the MPT51 20-mer products, aa 21–40 is probably ubiquitinated at the amino-terminal end since this peptide contains no lysine residue. It is unclear whether 20-mer products resist ubiquitination. However, the length seems to be a critical factor for processing because a 40-mer product, MPT51 1–40, which also contains no lysine residues, was successfully processed and presented to T cells (Fig. 3B). Further investigation is needed to resolve this length effect. It seems likely that the poor ubiquitination of 20-mer products has hampered the identification of T cell epitopes by the expression library system thus far.

Once the T cell epitope was defined within aa 21–40, we utilized three of the available epitope prediction programs to try to identify the most likely stretch of amino acids that contained the minimal T cell epitope. Of the six candidates that scored in the top three using each algorithm, one of these, aa 24–32, induced IFN-γ production in MPT51-immune spleen cells when stimulated with a P815 line expressing this 9-mer peptide. As expected, ubiquitination was not required for presentation of the 9-mer peptide (Figs. 5B and 6) (Uchijima et al., 1998). We have concluded that MPT51 has only one $CD8^+$ T cell epitope in BALB/c mice using this retroviral expression system. This is consistent with previous results obtained using a conventional overlapping peptide library composed of 20-mer peptides (Suzuki et al., 2004).

Various factors are involved in epitope selection, such as antigen processing and the T-cell repertoire, in addition to a peptide's affinity for MHC molecules. In general, the programs used can only predict the potential epitope candidates from the whole sequence. In fact, when the whole protein sequence was scanned by these programs, there were a number of other peptides that were predicted as having high scores, although we found no evidence of T-cell response to these regions of the protein. In some cases, peptides showing lower scores can be used to eliminate some of these possible candidates. However, there may be cases where peptides with lower scores could represent dominant or sub-dominant epitopes. Although the use of such programs may be useful in the selection of

potential peptide candidates for testing, all of these peptides should be tested individually to confirm their immunologic relevance.

It is possible that some of the transformants poorly expressed MPT51 fragments, but this seems unlikely since we employed G418-resistant stable transformants. In our experience, most of transformants that survive under G418 selection conditions expressed the transduced gene as well as the *neo* gene as have been reported by other investigators (Gubin et al., 1997; Ward et al., 2003). In fact, the majority of the transformants used in this study expressed their transduced MPT51 gene fragments well (Fig. 2B). The use of a protein expression tag, such as a His tag, may help in monitoring gene expression. However, we did not employ such a tag system because the addition of this tag to protein fragments had the potential to alter the processing of the protein and to change its recognition (Del Val et al., 1991; Mo et al., 2000).

The strategy described in this report has several advantages over other methods in identifying $CD8^+$ T cell epitopes. The retroviral expression library construction can be performed simply using PCR cloning and several oligonucleotides can be prepared at the same time to cover the entire protein. It is obvious that this approach has a certain cost saving over choosing to synthesize overlapping peptides, particularly if the protein is of a larger size. In addition, strategies that use synthetic 15- to 20-mer peptides may fail to detect a $CD8^+$ T cell response due to the necessity of trimming the peptide to the proper 8–10-mer length required for MHC class I presentation. In our method, protein fragments can be properly processed inside cells and loaded on the MHC class I naturally and is thus suitable for assessing a natural $CD8^+$ T cell response. It also appears effective for detecting epitopes produced by protein splicing. It has been shown that CTL against tumors recognize, in certain cases, antigenic peptides produced by post-translational protein splicing in the proteasome (Hanada et al., 2004; Vigneron et al., 2004). Such epitopes cannot be detected using a conventional peptide library. The present retroviral mapping system has the potential to detect such spliced epitopes.

One of the other impediments to T cell epitope characterization is the determination of the MHC class I restriction molecules. The present method circumvents this problem because the potential MHC class I molecules of interest can be co-transfected with the recognized T cell epitope. Using this co-transfection strategy, we identified H2-D^d as a restriction molecule for the MPT51 24–32 epitope. This finding allowed us to employ the H2-D^d/MPT51 24–32 tetramer to measure the size of the T cell population specific for MPT51 24–32 after immunization with a DNA vaccine encoding MPT51.

Our results allowed us to simplify the retroviral epitope detection system. It is possible to skip the 40-mer step and directly prepare DNA encoding ubiquitin-conjugated 20-mers spanning the candidate fragment, which means that this method could theoretically replace the synthesis of an overlapping conventional peptide library for $CD8^+$ T cell epitope mapping.

Taken together, our epitope mapping method using a retroviral expression system is comparable in efficiency and accuracy to a conventional peptide library for $CD8^+$ T cell epitope mapping and is much more cost-effective. Using this system, the preparation of a large peptide library or synthesis of numerous peptides predicted by program algorithms is not necessary. In conclusion, the use of this methodology could find a wide application in quickly identifying immunologically relevant $CD8^+$ T cell epitopes important in infectious diseases and cancer.

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Cytotoxic T-Lymphocyte-, and Helper T-Lymphocyte-Oriented DNA Vaccination

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ABSTRACT

DNA vaccines have advantages over other types of vaccines in that they can induce strong cellular immune responses, namely cytotoxic T lymphocytes (CTL) and helper T lymphocytes (Th). DNA vaccines are therefore considered a promising alternative to attenuated live vaccines in the field of infectious diseases. So far, various DNA vaccines have been generated and tried to induce a particular cellular immune response by virtue of recombinant DNA technology. DNA vaccines have been designed for efficient transcription and translation of target genes by a variety of strategies. Also, various DNA vaccine strategies for induction of specific CTL and Th have been reported by taking into consideration antigen presentation pathways and the strategies have been shown to be effective to elicit particular T-cell responses. In this paper, we have reviewed these strategies, including our study on epitope-specific T-cell induction by DNA vaccination against *Listeria monocytogenes* infection. From this review, it has been surmised that, to induce strong immune responses by DNA vaccines, the immunization route and the immunization regimen, such as heterologous "prime-boost" regimen, should also be considered.

INTRODUCTION

DNA VACCINATION IS A VACCINATION method by the direct inoculation of a eukaryotic expression plasmid encoding antigen molecules of interest into host animals. The vaccines have been intensively studied in the past decade. It was surprising to know the paper by Wolff and colleagues (1990) describing that direct intramuscular injection of plasmid DNA allows the expression of plasmid-encoded proteins in the tissues of injected mice, because even expression of plasmid DNA in cultured cells needs some expertise in transfection methodology, such as the calcium phosphate precipitation method or the liposome method, etc. Subsequently, Tang and colleagues (1992) showed that injection of plasmid DNA directly into the skin with gene gun bombardment effectively induces specific antibody production. These early important studies have been reviewed in Chattergoon *et al.* (1997) and Liu and Ulmer (2000). Among the various vaccination strategies tried so far, DNA vaccines have been shown to have advantages over other types of vaccines by inducing cellular immune responses, namely, cytotoxic T lymphocytes (CTL) and type 1 helper T lymphocytes (Th1), without utilizing live organisms. In addition, DNA vaccines offer several other advantages such as their relatively easy design and construction with enormous flexibility using recombinant DNA technology, their chemical stability, and relatively low cost.

We have reported to construct DNA vaccines able to elicit only a particular T-cell subset against T-cell epitopes derived from *Listeria monocyotogenes* and examined their efficacy. We review here strategies to induce specific T-cell subsets by naked DNA immunization with an emphasis on our results on DNA vaccines against *L. monocytogenes* as a model of DNA vaccination against intracellular pathogens.

OUTLINE OF DNA-MEDIATED IMMUNIZATION

Typical eukaryotic expression plasmids can be utilized for DNA vaccines. DNA vaccines are composed of (1) an antigen-encoding gene whose expression is driven by (2) a strong eukaryotic promoter such as cytomegalovirus immediate-early (CMV I.E.) promoter/enhancer, (3) a polyadenylation termination sequence such as the sequence derived from simian virus

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40 (SV40) or bovine growth hormone (BGH) gene, and (4) a prokaryotic selective marker such as ampicillin resistance gene to facilitate selection of *Escherichia coli* clones carrying the plasmid. In addition, plasmids for DNA vaccines should contain special nucleotide sequences for enhancing the immunogenicity, namely an unmethylated cytidine-phosphate-guanosine (CpG) dinucleotide with appropriate flanking regions. In mice, the optimal flanking region is composed of two 5′ purines and two 3′ pyrimidines (Van Uden and Raz, 2000). An ampicillin resistance gene contains the CpG, but a kanamycin resistance gene does not have it (Sato *et al.*, 1996). The CpG motif stimulates the innate immune system through Toll-like receptor 9 to produce a series of immunomodulatory cytokines such as interleukin (IL)-12 and interferon (IFN)-γ, which promote the development of Th1 cells (Krieg *et al.*, 1995; Klinman *et al.*, 1996; Roman *et al.*, 1997).

Major immunization methods for DNA vaccines tried so far are, (1) intramuscular injection into the hind leg quadriceps or tibialis anterior, (2) gene gun bombardment of DNA-coated gold particles into the epidermis, (3) intradermal DNA immunization (Raz *et al.*, 1994), and (4) topical application of DNA vaccines (Fan *et al.*, 1999) that have also been reported to be able to induce immunization effects. Furthermore, several "carrier"-mediated DNA vaccine administration methods have been reported such as (5) liposomes, (6) microparticle encapsulation, and (7) attenuated bacteria. These methods are briefly reviewed in Gurunathan *et al.* (2000).

In intramuscular immunization, primary cells that plasmid DNA is transferred into are myocytes. As myocytes are not professional antigen-presenting cells (APC), the mechanisms of DNA vaccines have been controversial, but bone marrow-derived APC has been suggested to be involved in antigen presentation in DNA vaccines (Corr *et al.*, 1996; Iwasaki *et al.*, 1997).

It is of particular interest that gene gun DNA immunization requires 100 to 1000 times less DNA than muscle DNA inoculation to generate equivalent antibody responses (Pertmer *et al.*, 1995, 1996). In addition, gene gun DNA immunization appeared to bring about highly reproducible and reliable results in antibody production and induction of specific CTL and IFN-γ production from immune splenocytes (Yoshida *et al.*, 2000). It has been suggested that muscle DNA immunization tends to raise predominant Th1 responses, while gene gun DNA immunization is apt to produce Th2 responses (Feltquate *et al.*, 1997). The difference is considered to be mainly due to the difference of plasmid amounts used for the vaccinations. This difference may affect (1) the amount of antigen produced from the plasmids and (2) the amount of CpG motif present in plasmid DNA vaccines.

DNA VACCINE DESIGN FOR INCREASING ANTIGEN LEVEL IN APC

Investigators have tried to optimize DNA vaccination by increasing the expression level of antigenic molecules in APC. A variety of factors affects the expression level. We would like to review strategies for enhancing antigen expression level in APC.

Choice of eukaryotic promoter

Cheng and colleagues (1993) assessed the activities of five viral and five cellular promoters in different rat tissues by using gene gun bombardment. Their results demonstrated that CMV I.E. enhancer/promoter activity was consistently the highest in each tissue. Hence, the promoter has been used intensively for DNA vaccines. For the specific expression in myocytes, desmin promoter, which works specifically in myocytes, was also used for DNA vaccination via intramuscular injection (Kwissa *et al.*, 2000). Promoters that are preferentially active in professional APC, like scavenger receptor gene promoter, MHC class II promoter or dectin-2 promoter (Zhi *et al.*, 1997; Corr *et al.*, 1999; Takashima and Morita, 1999, respectively) have been examined, but several reports showed that they did not induce satisfactory immune responses (Zhi *et al.*, 1997; Corr *et al.*, 1999).

Inclusion of the Kozak consensus translational initiation sequence

For the efficient translation of target genes, a Kozak consensus translational initiation sequence around ATG translation start codon (CCA/GCCATG) have been shown to be important (Kozak, 1987). An and colleagues (2000) suggested that the Kozak consensus sequence should be added in minigene DNA vaccine constructs. Strugnell and colleagues (1997) reported that the DNA vaccine construct for expression of *Chlamydia trachomatis* major outer membrane protein (MOMP) with the most optimal Kozak sequence ("A" at −3 position relative to the initiation codon) showed the high expression of MOMP protein when transfected into Cos-7 cell line. Nevertheless, the plasmid injected into mice did not result in satisfactory antibody production. In addition, any "ATG" sequences in the 5′-untranslated region of antigen genes should be removed because the sequence may work as the translation start codon. The distance between the promoter and the open reading frame would also affect the expression level of antigens.

Codon optimization

Interspecies difference of codon usage is one of the major obstacles for effective induction of specific immune responses against pathogens by DNA vaccination. When genes derived from pathogens such as bacteria, protozoa, and some viruses, codon usage is one of problems for the expression in eukaryotic cells. We constructed a plasmid DNA vaccine harboring a wild-type DNA sequence of a dominant CTL epitope of *L. monocytogenes* derived from hemolysin, listeriolysin O (LLO), LLO 91–99 (p91wt), and then tried immunization of mice with the DNA vaccine by intramuscular injection. However, we could not clearly induce LLO 91–99-specific CTL in BALB/c mice (Uchijima *et al.*, 1998). One of the reasons for the failure of the induction may be the difference of the codon usage between mammalian cells and *L. monocytogenes*. *L. monocytogenes* genome is highly A+T-rich. In contrast, the mammalian genome is G+C-rich. This difference may affect the efficiency of *L. monocytogenes* gene expression in mammalian cells. So, we constructed a DNA vaccine using the LLO 91–99 gene, whose codons were optimized to those of the mammalian cells

(p91mam). The DNA vaccine gave an excellent CTL induction by intramuscular immunization (Uchijima *et al.*, 1998). We further evaluated the "codon optimization effect" on CTL induction by the DNA vaccine (Nagata *et al.*, 1999). In that study, we analyzed in mammalian cultured cells the translation efficiency of several genes composed of different levels of optimization to mammalian cells, but encoding an identical CTL epitope derived from *L. monocytogenes*, LLO 91–99 or a murine malaria parasite, *Plasmodium yoelii*, PyCSP 281–290, and showed that the codon optimization level of the genes is not precisely proportional to, but does correlate well, with the translation efficiency in mammalian cells. The results also correlated well with the induction level of specific CTL response in vivo (Nagata *et al.*, 1999; Fig. 1). In that study, we used the relative synonymous codon usage (RSCU) and the codon adaptation index (CAI) values as the indices of the codon optimization level. These values have been explained by Sharp and Li (1987). The RSCU values of codons used in *L. monocytogenes* and the malaria parasite (*Plasmodium* spp.) showed the opposite relationship to the RSCU values of codons used in mice and humans, indicating that native codons frequently used in these organisms are rarely used in mice and humans. However, such a relationship is not necessarily applicable in all pathogens. For example, RSCU values of codons used at high frequency in *Mycobacterium tuberculosis* genes are quite similar to those in *Mus musculus* genes (Nakamura *et al.*, 2000). In such a case, codon optimization for DNA vaccine constructs may not be necessary.

A similar effect of codon optimization was noted in a murine DNA immunization model against human immunodeficiency virus type 1 (HIV-1) infection, by using the genes encoding HIV-1 gp120 (André *et al.*, 1998), gp160 (Vinner *et al.*, 1999) or gag (Deml *et al.*, 2001), and also in a murine malaria DNA vaccine encoding *Plasmodium falciparum* merozoite protein (Narum *et al.*, 2001).

Facilitation of intercellular spreading of antigens

To facilitate intercellular spreading of antigens in vaccinated animals, Hung and colleagues (2001) reported a unique interesting approach using VP22 protein. VP22 is a unique herpes simplex virus-1 protein that has been demonstrated the remarkable property of intercellular transport and shown to be capable of facilitating the spread of fused proteins to surrounding cells (Eliott and O'Hare, 1997). Hung and colleagues constructed a DNA vaccine of VP22-human papillomavirus type 16 E7 chimeric protein and showed that the DNA vaccine successfully spread the fused protein *in vivo* and enhanced MHC class I presentation of the antigen.

Targeting DNA to APC and enhancing antigen presentation ability of APC

Induction of specific T cells requires antigen presentation by professional APC. Therefore, introduction of target genes specifically into professional APC by DNA immunization may be useful for inducing T cells efficiently. To realize this intention, the gene gun immunization method would be the best, as this method allows the genes to enter dermal dendritic cells and Langerhans cells directly.

Some investigators have reported elegant DNA vaccine strategies for the localization of antigens to professional APC efficiently. Boyle and colleagues (1998) reported that DNA vaccines for human immunoglobulin attached to CTLA-4 molecule, which allows targeting the antigen to APC, enhanced antibody production against the immunoglobulin, although the same approach did not enhance CTL responses (Deliyannis *et al.*, 2000). In another approach, You *et al.* (2001) evaluated a DNA vaccine for a model hepatitis B virus *e* antigen fused to an IgG Fc fragment. They showed that the secreted fusion proteins are efficiently captured and processed by dendritic cells via receptor-

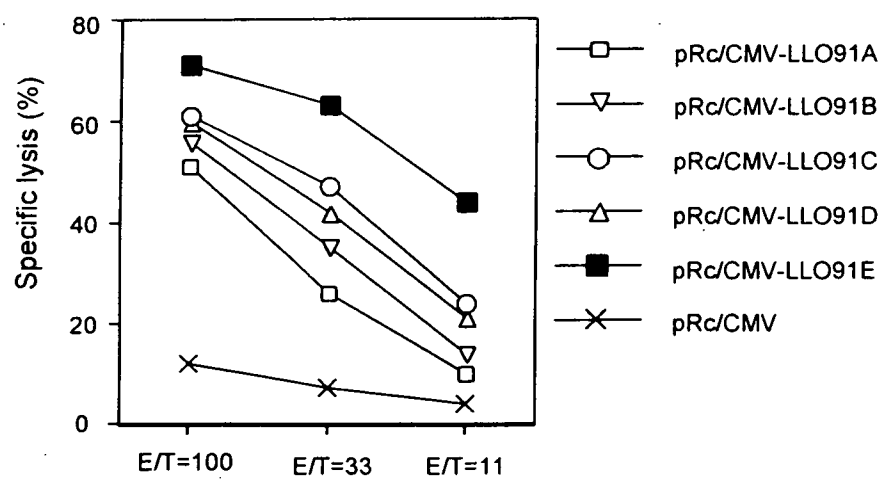

FIG. 1. Codon optimization effect on specific CTL induction by DNA vaccination. BALB/c mice were immunized by gene gun with several plasmids for expression of LLO 91–99, whose codons were optimized to the mouse to the different extent, pRc/CMV-LLO91 **A, B, C, D,** or **E.** Immune spleen cells were stimulated *in vitro* with LLO 91–99-pulsed syngeneic splenocytes for 5 days and subjected to CTL assay. The percentage of the specific lysis was determined using BALB/3T3 cells pulsed with LLO 91–99 peptide as target cells (reproduced from Nagata *et al.*, 1999).

mediated endocytosis and then presented to the major histocompatibility complex (MHC) class I and class II molecules.

Efficient antigen presentation requires accessory molecules expressed on APC. Latouche and Sadelain (2000) showed that B7.1, ICAM-1, and LFA-3 molecules are most important for antigen presenting capacity. Coadministration of these accessory molecules will improve the immunization effects of DNA immunization. In an analogous manner, the activation of T cells was facilitated by coadministration of plasmids encoding CD40 ligand and/or cytokines (Gurunathan *et al.*, 1998).

ANTIGEN PROCESSING AND PRESENTATION REQUIRED FOR INDUCTION OF T-CELL SUBSETS (CTL AND Th)

The types of effective immune responses against infectious diseases depend on the location of pathogens responsible. For example, extracellularly located pathogens are, in general, vulnerable to antibody-mediated effector mechanisms. On the other hand, protection against intracellularly located pathogens depends on induction of specific cell-mediated immunity (Kaufmann, 1993; Hess *et al.*, 2000). Two major arms of cellular immunity come into play in the protection. CTL is a main effector against pathogens located in the cytoplasm of host cells, such as viruses, *Rickettsia* spp. or *L. monocytogenes*, while Th1 plays a pivotal role in the protection against infections with intracellular pathogens located in vacuolar compartments, such as *Mycobacteria* spp. or *Salmonella* spp. Therefore, induction of effective resistance to infection depends on vaccines being capable of eliciting certain effectors. Both cellular immune responses have been shown to be effectively induced with DNA vaccines (Chattergoon *et al.*, 1997; Donnelly *et al.*, 1997; Alarcon *et al.*, 1999; Gurunathan *et al.*, 2000; Shedlock and Weiner, 2000).

Both CTL and Th have T-cell receptor molecules on their surface in common, but CTL and Th in general have CD8 molecules and CD4 molecules on their surface, respectively. $CD8^+$ CTL are presented antigens (antigenic peptides) in association with MHC class I molecules on the surface of APC, and $CD4^+$ Th are presented antigens in association with MHC class II molecules. Therefore, efficient induction of CTL and Th requires efficient presentation of antigenic molecules through MHC class I and class II antigen processing and presentation pathway, respectively.

MHC class I molecules have been shown to be expressed in almost all somatic cells except for neurons and germ cells. To prime CTL, antigenic peptides must be presented on MHC

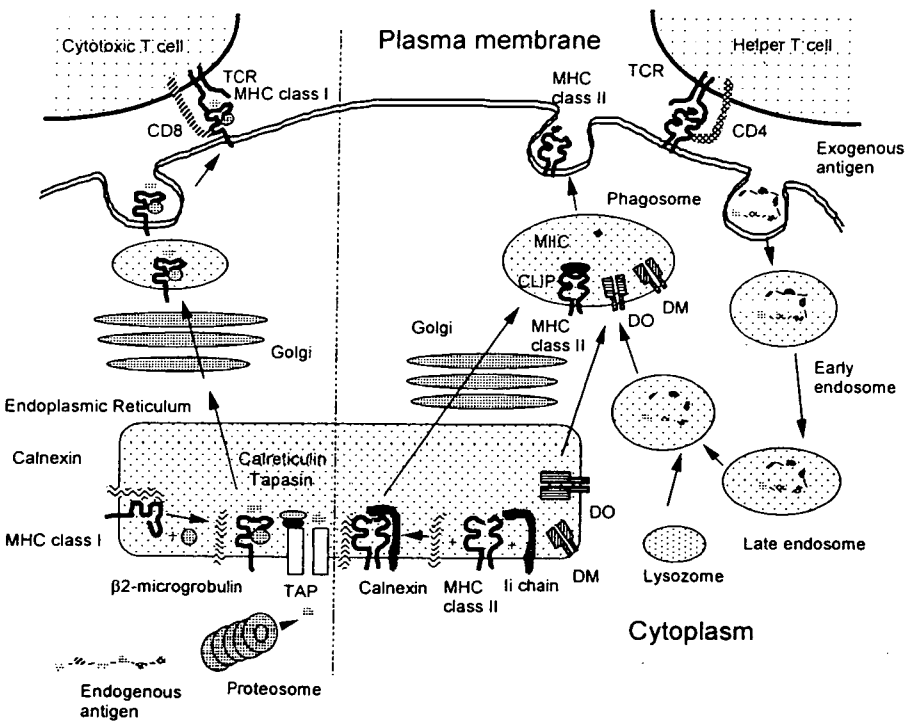

FIG. 2. Antigen processing and presentation pathways through MHC class I and class II molecules. Endogenous antigens are thought to be degraded by large, ATP-dependent proteasome complex. The resulting peptides are then translocated into the lumen of the ER by TAP transporters. In the ER, antigenic peptides bind to the groove of MHC class I molecules. The peptide–MHC class I complexes are transported through the Golgi to the cell surface. Exogenous antigens are phagocytosed by phagocytes such as macrophages. Then, the antigens are degraded into peptides in endosome/lysosome compartments. MHC class II molecules associate with Ii molecules in the ER and the complex moves to endosome/lysosome compartments. Antigenic peptides bind to MHC class II molecules in the compartment named MIIC with the help of DM and DO molecules. The peptide–MHC class II complexes are then displayed on the cell surface.

class I molecules on the surface of professional APC, which possess special accessory molecules. In general, proteins located in the cytoplasm of the APC (endogenous antigens) are processed with the proteasome complex and selected peptides go into the endoplasmic reticulum (ER) through transporters associated with antigen processing (TAP) molecules. Antigenic peptides are supposed to bind to the groove of MHC class I molecules in the ER. Then, they go to the cell surface and are presented to $CD8^+$ T cells [Fig. 2; reviewed in Pamer and Cresswell (1998) and Rock and Goldberg (1999)]. On the other hand, MHC class II molecules are expressed only in professional APC having specific antigen presenting capacity, such as macrophages, dendritic cells, and B cells. Basically, MHC class II molecules are able to present antigenic peptides derived from the outside of the APC (exogenous antigens). Exogenous proteins are phagocytosed into APC and localized in the vesicles called phagosomes separated from the cytoplasm by lipid bilayer membranes. Phagosomes then fuse with lysosomes, which are also vesicular compartments full of peptidases. After the fusion, exogenous proteins are degraded into smaller pieces of peptides. MHC class II molecules associate with invariant chain (Ii) molecules in the ER and the complex goes to the endosomes by virtue of endosomal targeting signals located in the cytoplasmic regions of Ii molecules and also of MHC class II molecules. In the endosomes, Ii molecules are gradually degraded and only a small portion designated class II associated Ii peptides (CLIP), which is located in the groove of MHC class II molecules, remained. Then, antigenic peptides exchange with CLIP with the help of MHC class II-like molecules, H2-DM and H2-DO (mice), or HLA-DM and HLA-DO (humans) under the influence of local acidic pH and finally go into the groove of MHC class II molecules. They are then finally presented to $CD4^+$ T cells on the surface of APC [Fig. 2; reviewed in Germain (1999); Bryant *et al.* (2002)]. Targeting of antigens into endosomal compartments is a key factor to facilitating induction of antigen-specific $CD4^+$ T cells.

T-CELL RESPONSES TO *LISTERIA MONOCYTOGENES* INFECTION—DNA VACCINE STUDY AGAINST INTRACELLULAR PATHOGENS

We have been working on DNA vaccines against *L. monocytogenes* as a model of intracellular bacteria. *L. monocytogenes* is a Gram-positive facultative intracellular bacterium. Murine *L. monocytogenes* infection system has been studied as a good model system for intracellular bacteria infection (Cossart and Mengaud, 1989; Kaufmann, 1993). The bacterium has been known to induce MHC class I-restricted $CD8^+$ T-cell responses in addition to MHC class II-restricted $CD4^+$ T-cell responses since the bacterium is capable of escaping from phagocytic vesicles into the cytoplasm of the host cells, thereby introducing the bacterial proteins into the MHC class I antigen processing pathway. Both $CD8^+$ CTL and $CD4^+$ Th1 have been shown to be amplified at listerial infection and to play a critical role in protective immunity in experiments of depletion and adoptive transfer of specific T-cell subsets (Kaufmann *et al.*, 1985; Czuprynski and Brown, 1990; Sasaki *et al.*, 1990) or by analyses of mutant mice with a genetic defect in β2-microglobulin or H2-Aβ gene (Roberts *et al.*, 1993; Ladel *et al.*, 1994).

So far, several T-cell epitopes (MHC-binding antigenic peptides) in listerial antigens have been reported (Fig. 3). Pamer and colleagues (Pamer, 1997; Busch *et al.*, 1999) have reported four different *L. monocytogenes* epitopes presented by MHC class I ($H2\text{-}K^d$) molecules to CTL; those are listeriolysin O (LLO) 91–99, p60 (murein hydrolase) 217–225, p60 449–457, and mpl (metalloprotease) 84–92. Two of these four epitopes, LLO 91–99 and p60 217–225, have been demonstrated to induce dominant immune responses. First identified MHC class II binding peptide in *L. monocytogenes* is LLO 215–226, an $H2\text{-}E^k$ binding peptide (Ziegler *et al.*, 1994; Safley *et al.*, 1995). Then, p60 301–312 was identified as an $H2\text{-}A^d$ binding peptide (Geginat *et al.*, 1998). Recently, Geginat *et al.* (2001) tried

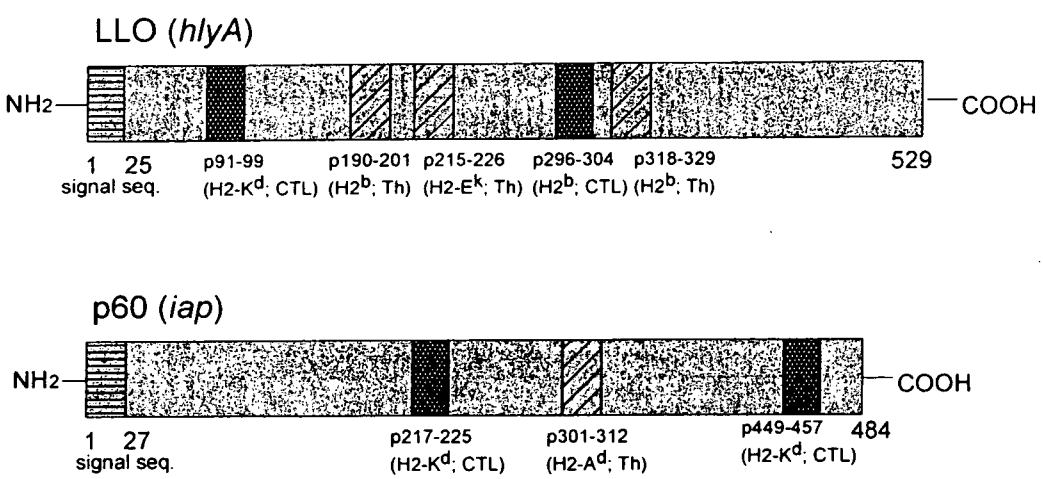

FIG. 3. Major protective antigens of *Listeria monocytogenes*, listeriolysin O (LLO), and p60. LLO and p60 molecules of *L. monocytogenes* have been shown to be major protective antigens. So far, several $CD8^+$ CTL and $CD4^+$ Th epitopes in these antigens have been reported.

to identify $CD4^+$ and $CD8^+$ T-cell epitopes in LLO and p60 molecules based on the screening of peptide spot libraries with splenocytes derived from *Listeria*-infected BALB/c and C57BL/6 mice. They confirmed all known $CD4^+$ and $CD8^+$ T cell epitopes in LLO and p60 molecules and additionally identified six new $H2^d$-, and six new $H2^b$-restricted T cell epitopes, containing $H2^b$-restricted LLO 190–201, LLO 318–329 ($CD4^+$ T-cell epitopes) and LLO 296–304 ($CD8^+$ T-cell epitopes) (Fig. 3). The adoptive transfer of LLO 91–99-specific $CD8^+$ CTL (Harty and Bevan, 1992) or p60 301–312-specific $CD4^+$ Th (Geginat *et al.*, 1998) conferred protection against lethal listerial infection, suggesting that induction of T cells specific to these T-cell epitopes is a prerequisite for protection against listerial challenge.

CTL-ORIENTED DNA VACCINES

A variety of studies have been conducted on the efficient induction of CTL of a particular specificity and is summarized in Figure 4.

CTL-Epitope minigene DNA vaccines and the effect of the addition of the leader sequence on CTL induction

As mentioned previously, we have demonstrated that the minigene DNA vaccine, encoding only a dominant CTL epitope of *L. monocytogenes*, LLO 91–99, was effective for inducing CTL *in vivo* by gene gun-mediated DNA immunization (Uchijima *et al.*, 1998; Nagata *et al.*, 1999). These results suggest that the DNA vaccine plasmids are directly incorporated in APC and that the APC present target peptides to T cells by DNA immunization, although Cho *et al.* (2001) suggested that cross-priming is a predominant mechanism for inducing $CD8^+$ T-cell responses in gene gun DNA immunization. Some CTL epitopes were reported to be modified to have more immunogenic capacity by substituting several amino acid residues (epitope enhancement; Berzofsky, 1993). Berzofsky *et al.* (2001) suggested that epitope sequences can be modified in three ways: (a) for increasing the affinity of binding to an MHC molecule; (b) for increasing the affinity of the peptide–MHC complex for the T-cell antigen receptor; or (c) for achieving a more broadly crossreactive T-cell responses.

Ciernik *et al.* (1996) added a DNA sequence encoding adenovirus E3 leader sequence to mutant p53 minigene (more precisely, not the minimal CTL epitope, but CTL epitope-covering portion) and constructed the minigene plasmid and showed that the plasmid DNA vaccination is capable of eliciting CTL against mutant p53 efficiently. Addition of the leader sequence will localize the target polypeptide in the ER, bypassing the need for the TAP transporter. Subsequently, the MHC class I binding peptide (the CTL epitope) in the mutant p53 molecule would efficiently bind to MHC class I molecules there. They reported that the addition of the leader sequence dramatically induced the specific CTL activity. As mentioned earlier, they used the mutant p53 peptide, which contains 18 amino acids, instead of the minimal CTL epitope in it. The peptide has to be trimmed before binding to MHC class I molecules in the ER. In such a case, accumulation of the peptide in the ER by virtue of the leader sequence will facilitate the binding of the peptide and MHC class I molecules in the ER. However, the LLO 91–99 peptide used in our study (Uchijima *et al.*, 1998; Nagata *et al.*, 1999) is a 9-mer peptide. In this case, further trimming is not required for binding to MHC class I molecules. The difference would explain the

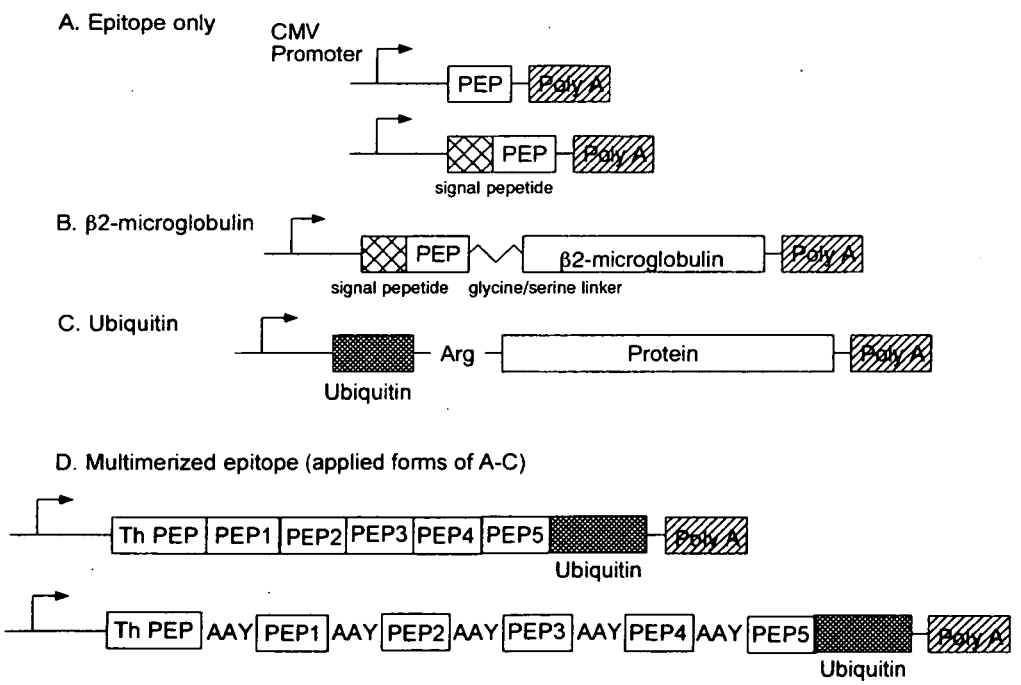

FIG. 4. Schematic presentation of several CTL-oriented DNA vaccines. Gene structures of CTL-oriented DNA vaccines are shown. See following references, Uchijima *et al.* (1998), Ciernik *et al.* (1996) for (**A**), Uger and Barber (1998) for (**B**), Wu and Kipps (1997) for (**C**), and Velders *et al.* (2001) for (**D**).

reason why CTL minigene DNA vaccine induces specific CTL efficiently without the addition of the leader sequence in our studies, although Iwasaki *et al.* (1999) reported that the addition of the leader sequence still enhances the magnitude of the CTL responses by a minimal CTL epitope DNA vaccine with both intramuscular and gene gun-mediated administration.

Fusion with β2-microglobulin

Uger and Barber (1998) prepared an expression plasmid of influenza virus nucleoprotein (NP) 366–374 (H2-D^b-restricted CTL epitope) fused with β2-microglobulin molecule by a glycine/serine short linker. They introduced the plasmid into a murine cultured cell line, and showed the efficient loading of the CTL epitope on MHC class I molecules. Their study is not an *in vivo* study, but the plasmid may be usable in DNA vaccine for efficient CTL induction.

Ubiquitination of target genes

In general, intracellular protein antigens are processed into the peptides for antigen presentation via MHC class I molecules, as mentioned previously. The peptides have been reported to be generated mainly via the ubiquitin (Ub)-proteasome pathway although the Ub-independent pathways have also been suggested (Murakami *et al.*, 1992; Jariel-Encontre *et al.*, 1995). Ub is a 76-amino acid peptide involved in controlling the normal protein intracellular turnover in the cytoplasm of eukaryotic cells. Ubiquitination of cellular proteins occurs in lysine residues of the proteins in an ATP-dependent manner, and the ubiquitinated proteins have been reported to be sensitive to degradation by proteasomes. The identity of the N-terminal residue of proteins is a determinant of whether the proteins are sensitive or resistant to degradation by proteasomes (N-end rule; Varshavsky, 1992). For example, if the N-terminal residue is methionine, the protein is stable, and if the residue is arginine, the protein is destabilizing.

So far, DNA vaccines that enhance the ubiquitin-proteasome degradation of target antigens have been reported. Wu and Kipps (1997) prepared an expression plasmid for chimeric β-galactosidase (β-gal)-*LacI* partial segment containing two lysine residues (served as a substrate of ubiquitination) and also an arginine residue was added in the N-terminal region (pUb-Arg-*lacZ*). They used this fusion plasmid as a DNA vaccine. This linear fusion of one Ub to chimeric β-gal-*LacI* molecule is supposed to be cleaved after the last residue of the Ub portion *in vivo* by Ub-specific processing proteases (Backmair *et al.*, 1986). After the cleavage, the Arg residue becomes the utmost N-terminal residue of the processed molecule, which is subsequently subjected to ubiquitination and degradation. As expected, immunization with the DNA vaccine showed efficient induction of β-gal-specific CTL. pUb-Arg-*lacZ*-transfected P815 cells (a murine mastocytoma cell line) did not have β-gal enzymatic activity, probably due to degradation by proteasomes. Whitton's group (Rodriguez *et al.*, 1997; Whitton *et al.*, 1999) also showed similar results by using expression plasmids for ubiqitinated NP derived from lymphocytic choriomeningitis virus. These results indicate that the strategy of antigen ubiquitination is useful to generate enhanced CTL activity and to reduce the production of antigen-specific antibodies. However, Fu *et al.* (1998) reported that the modifications of NP DNA vaccine with ubiquitin conjugation did not affect their ability to induce specific CTL responses, suggesting ubiquitin conjugation alone does not guarantee the improved targeting of endogenously synthesized antigens to MHC class I antigen processing pathway.

Injection of a single CTL epitope minigene DNA will generate a single CTL epitope peptide, which is supposed to enter the ER through TAP transporters. Our results (Uchijima *et al.*, 1998; Nagata *et al.*, 1999) indicated such a single minigene DNA immunization without any modification induced specific CTL efficiently. Interestingly, Rodriguez *et al.* (1998) showed even such a minigene DNA immunization improved by ubiquitination in terms of induction of high frequency of memory CTL, suggesting that ubiquitination allows more effective delivery of the minigene to the proteasome. They used a somewhat longer minigene encoding 32 amino acid residues covering two MHC class I binding peptide-encoding regions. In such a case, further trimming of peptides is necessary for the binding on MHC class I molecules as in the aforementioned minigene DNA vaccine construct described in Ciernick *et al.* (1996). This may be a reason why ubiquitination improved CTL induction after their minigene DNA vaccination. Our preliminary results show that such minigenes whose translation products do not have the exact size that fits in the groove of MHC class I molecules (8-, to 9-mer), but have some extra length, very inefficiently present the antigen to T cells (Aoshi, unpublished observation). We speculate that polypeptides bigger than minimal antigenic peptides, but smaller than the size of common proteins (e.g., around 20-mer peptides) are very resistant to proteasomal degradation. Proteasome complex may be facilitated for degradation of unfolded or misfolded large proteins, but may not be used for degradation of such artificially small peptides. In such a case, the ubiquitination of such small peptides dramatically increase the proteasomal degradation of the peptides.

Another intriguing strategy for enhancing proteasomal localization of antigens was reported by Hung and colleagues (2003). The centrosome has been shown to be a subcellular compartment rich in proteasomes (Antón *et al.*, 1999). They reported a DNA vaccine for a chimeric molecule composed of γ-tubulin (an established centrosomal marker) and human papillomavirus type 16 E7 (a model tumor antigen). They showed that the DNA vaccination targeted the antigen to centrosomal compartments and showed that the DNA vaccination induced enhanced MHC class I presentation and a marked increase in the number of E7-specific $CD8^+$ T-cell precursors.

Fusion with a carrier protein

Describing one particular approach for the efficient induction of $CD8^+$ T-cell subset, Wolkers *et al.* (2002) recommended a carboxyl-terminal fusion of CTL epitope to a carrier protein of foreign origin. They constructed their DNA vaccines encoding a carboxy-terminal fusion of either H2-D^b-restricted CTL epitope, NP 366–374 derived from influenza virus, or H2-D^b-restricted E7 49–57 derived from human papilloma virus to green fluorescent protein (GFP) and showed that the DNA vaccines induced much larger clonal size of antigen-specific $CD8^+$ CTL by intramuscular immunization of these DNA vaccines compared with the clonal size induced by these epitope minigene DNA vaccination. The purpose of the GFP fusion strategy was

the provision of $CD4^+$ T-cell help through recognition of GFP-encoded $CD4^+$ T cell epitopes. Maecker *et al.* (1998) also showed that CTL induction by both intramuscular and intradermal DNA administration is dependent upon the generation of $CD4^+$ T-cell help via a class II MHC-dependent pathway. Our results showed that CTL minigene DNA vaccination with gene gun induced specific CTL without any $CD4^+$ T-cell help (Yoshida *et al.*, 2001). We speculate that the route of naked DNA immunization is critical for requirement of CD4 T-cell help. So far, intramuscular or intradermal DNA administration was carried out in many reports which show that T-cell help is important for CTL induction. On the other hand, gene gun-based DNA immunization was carried out in many reports which show that CTL induction occurred independent of CD4 T-cell help.

Multimerized CTL epitope DNA vaccines

As a corollary of a single CTL epitope minigene DNA vaccination, several reports tried multimerized CTL epitope DNA vaccines (polyepitope DNA vaccines). The polyepitope vaccine was first evaluated by Whitton *et al.* (1993). They generated a recombinant vaccinia virus system for expression of CTL–epitope minigenes tandemly fused in a "string-of-beads" manner and showed that this "string-of-beads" vaccine can induce CTL specific to each different epitope and protect vaccinated animals against infections. Subsequently, Thomson *et al.* (1998b) constructed a DNA vaccine plasmid containing 10 contiguous minimal CTL epitopes, which were restricted by five MHC alleles and derived from five viruses (influenza virus, adenovirus, murine cytomegalovirus, Sendai virus, and lymphocytic choriomeningitis virus), a murine malaria parasite (*Plasmodium berghei*), and a tumor model antigen (ovalbumin). They injected mice with the plasmid by intramuscular injection or gene gun-mediated intradermal injection. They showed that the DNA vaccination successfully induces each epitope-specific CTL activity. Results of our single CTL–epitope DNA vaccine indicated that a single dominant CTL epitope is sufficient for the induction of protective immunity (Yamada *et al.*, 2001) suggesting that the selection of the most dominant CTL epitope for each pathogen is critical for the efficacy of DNA vaccines.

Although some reports suggest that flanking sequences of a CTL epitope are important for the precise processing of the CTL epitope *in vivo* and that some CTL epitopes will interfere with other epitope function (Del Val *et al.*, 1991), a majority of reports showed that immunization with multimerized CTL–epitope DNA without any spacer successfully induces CTL specific to each CTL epitope. But, some reports (e.g., Velders *et al.*, 2001) suggest the importance of defined flaking sequences around epitopes and addition of ubiquitin. Ishioka *et al.* (1999) evaluated minigene DNA vaccines encoding multiple HLA-restricted CTL epitopes employing HLA class I transgenic mice. Such studies are useful as pilot experiments to evaluate DNA vaccines before progressing to studies on human subjects.

HELPER T-CELL ORIENTED DNA VACCINES

A variety of studies have been performed on the efficient induction of Th of a particular specificity and is summarized in Figure 5.

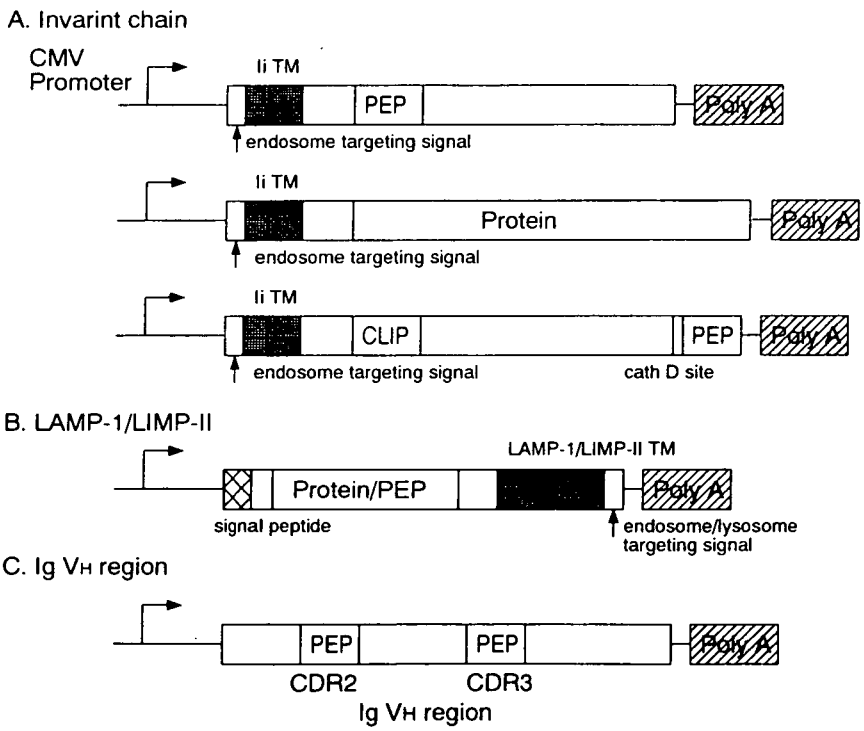

FIG. 5. Schematic presentation of several Th-oriented DNA vaccines. Gene structures of reported Th-oriented DNA vaccines are shown. See following references, for example, Nagata *et al.* (2001, 2002), Toda *et al.* (2002), van Bergen *et al.* (1997) for (**A**), Thomson *et al.* (1998a), Rodriguez *et al.* (2001) for (**B**), and Casares *et al.* (1997a, 1997b) for (**C**).

Th epitope/CLIP-exchanged DNA vaccines

Plasmid DNA is supposed to enter directly into APC, especially in gene gun-based vaccination. So, basically, the antigen presentation system through MHC class I molecules may be a primary antigen presentation route for DNA vaccination. However, efficient MHC class II antigen presentation is indispensable for efficient induction of $CD4^+$ helper T cells. Several groups have reported that MHC class II-positive cultured cells transfected with Ii cDNA, in which the CLIP region of the Ii molecule was replaced with a Th epitope of interest, efficiently stimulate specific T-cell lines (van Bergen *et al.*, 1997; Malcherek *et al.*, 1998; Fujii *et al.*, 1998, and reviewed in van Bergen *et al.*, 1999). We applied this strategy for Th-oriented DNA vaccines. We constructed the recombinant Ii DNA vaccine for OVA323-336 (Nagata *et al.*, 2001) or LLO 215–226 (Nagata *et al.*, 2002) Th epitope and evaluated the immunogenicity of the DNA vaccines. We observed the epitope-specific T-cell proliferation and IFN-γ production by spleen cells derived from the recombinant Ii DNA vaccines (Fig. 6). Some mice immunized with OVA 323–336-Ii DNA vaccine induced OVA 323–336-specific antibodies in the sera as well as the epitope-specific $CD4^+$ T cells (Nagata *et al.*, 2001). Recently, DNA vaccines of this type have been reported from other laboratories (van Tienhoven *et al.*, 2001; Toda *et al.*, 2002).

Utilization of endosomal/lysosomal targeting signals

Wu *et al.* (1995) reported a vaccine strategy for endogenous MHC class II presentation of antigens by taking advantage of the lysosome-associated membranous protein-1 (LAMP-1) molecule with the vaccinia virus system. The molecule is a type-1 transmembrane protein located predominantly in lysosomes and late endosomes involving the MHC class II antigen processing pathway. The cytoplasmic domain of LAMP-1 contains the amino acid sequence, Tyr-Gln-Thr-Ile, which is important for endosomal/lysosomal protein targeting. *In vitro* activation of Epstein-Barr virus-, and influenza virus-specific $CD4^+$ memory CTL was successfully demonstrated by infection of cultured cells with the recombinant vaccinia viruses for the polyepitope–LAMP-1 fusion proteins (Thomson *et al.*, 1998a). DNA immunization using this strategy has been examined for induction of a specific $CD4^+$ T-cell subset. Ji *et al.* (1999) showed that targeting human papillomavirus type 16 E7 to the endosomal/lysosomal compartments by gene-gun immunization enhances the antitumor immunity enough to protect mice against the challenge from virus protein-expressing tumors and to eradicate the preexisting tumor cells. We also tried a DNA vaccine for a chimeric molecule, where an antigenic peptide (LLO 215–226) was fused with the endosomal/lysosomal targeting signal in the LAMP-1 molecule. We observed that the LLO 215–226–LAMP-1 DNA vaccine gave induction of the epitope-specific Th induction and par-

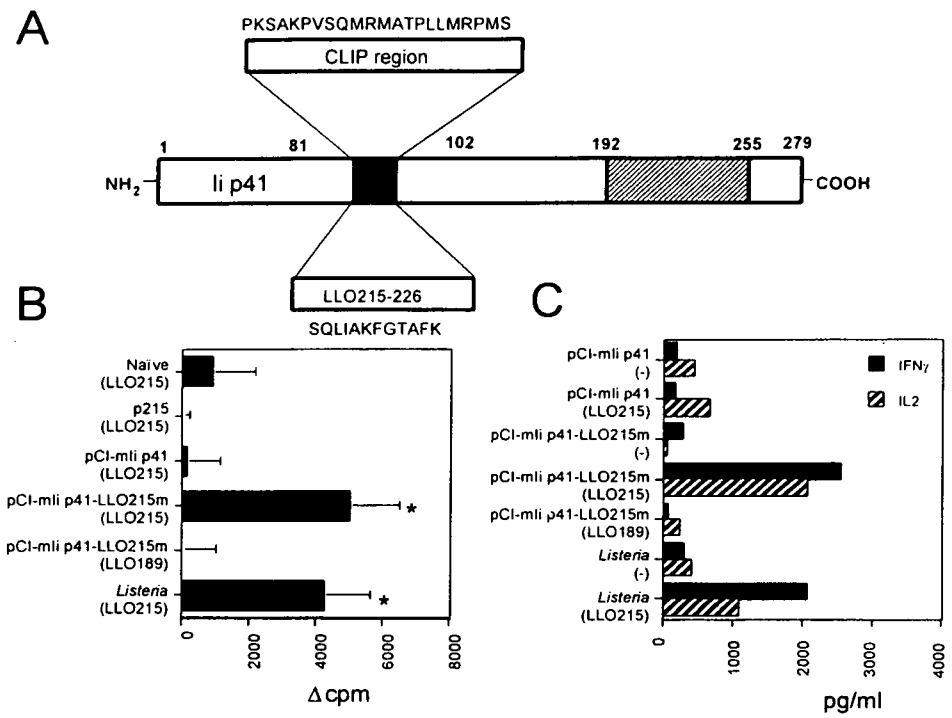

FIG. 6. Induction of LLO 215–226-specific Th by vaccination of plasmid for CLIP-replaced Ii molecule. (**A**) Schema of CLIP/LLO 215–226-replaced Ii molecule (mIi p41–LLO215m) whose expression plasmid was used for DNA vaccine. (**B**) LLO 215–226-specific splenocyte proliferation. C3H/He mice were immunized with the plasmid by gene gun bombardment four times. Immune spleen cells were harvested and cultured *in vitro* with indicated peptides (shown in parenthesis) for 2 days and then [^{3}H] thymidine was added for the last 12 h. Asterisks indicate statistical significance ($P < 0.001$) compared with the value of naive mice. (**C**) LLO 215–226-specific IFNγ and IL-2 production by immune spleen cells. The immune spleen cells were cultured with indicated peptides (shown in parenthesis) for 4 days (for IFN-γ) or 1 day (for IL-2). IFN-γ and IL-2 concentrations in the supernatants were qualified by sandwich enzyme-linked immunosorbent assay (modified figures of Nagata *et al.*, 2002).

tial protection against *L. monocytogenes* challenge, although the magnitude of the immunogenicity was somewhat lower than that by LLO 215–226/CLIP-replaced Ii DNA vaccine (Uchiyama *et al.*, 2002; Fig. 7). Vidalin *et al.* (1999) tried a DNA vaccine utilizing hepatitis C core protein–LAMP-1 fusion protein. Unfortunately, induction of the hepatitic C virus-specific T cells failed. They used a plasmid encoding the full-length hepatitis C virus core protein for the immunization, in which case the amounts of the MHC class II binding peptides produced would be low, as the protein must be further subjected to antigen processing to produce the peptides.

Recently, Rodriguez *et al.* (2001) reported successful CD4$^+$ T-cell induction by immunization of Th–epitope minigene plasmid DNA by utilizing the lysosomal targeting signal located in the carboxyl terminal tail of the lysosomal integral membrane protein-II (LIMP-II). They showed that the immunological consequences varied depending on the Th–epitope examined. As a result, a LAMP-1 or LIMP-II DNA vaccine may be useful for the targeting of proteins as well as peptides to the endosomal/lysosomal compartments, whereas the CLIP-replaced type of Ii DNA vaccine can afford to deliver only a small peptide, Th epitope, in theory. However, the immunological consequences of LAMP-1 or LIMP-II DNA vaccine may be weaker than the CLIP-replaced type of Ii DNA vaccine, and will also depend on target genes, as suggested in Rodriguez *et al.* (2001).

Utilization of a carrier protein structure-recombinant immunoglobulin molecule

Casares *et al.* (1997a, 1997b) reported on a DNA vaccine utilizing a recombinant immunoglobulin molecule, where the complementarity determining region (CDR) 3 and CDR2 regions of the heavy chain variable region were exchanged with an immunodominant Th epitope (HA 110–120) and a major B-cell epitope (HA 150–159) of influenza virus, respectively. They showed that immunization of mice with the DNA vaccine successfully induced influenza virus-specific Th and antibodies. This approach used a structurally rigid immunoglobulin molecule as the backbone molecule for the DNA vaccine.

Effect of the leader sequence on CD4 Th induction

Akbari *et al.* (1999) showed that vaccination with a DNA construct encoding the nonsecreted form (without the leader sequence) of the fifth component of the complement (C5) protein induces strong, long-lived CD4$^+$ T-cell responses. They showed that a small number of dendritic cells take up the plasmid DNA and migrate to the draining lymph nodes, and suggested that these dendritic cells are necessary for induction of long-lived CD4$^+$ T-cell responses. CD4$^+$ T-cell responses are generally thought to be induced by antigen presentation of "exogenous antigens" by dendritic cells. But this report showed that vaccination with a DNA construct encoding a protein that is not secreted is capable of inducing strong, long-lived CD4$^+$ T-cell responses.

Use of altered peptide ligand to increase peptide affinity for MHC molecules

Alexander *et al.* (1994) reported an artificial Th epitope that is capable of being presented on multiple HLA-DR molecules, by inserting anchor residues for diverse HLA-DR molecules in a polyalanine backbone and charged or bulky residues in the positions interacting with the TCR. This strategy may be applied for construction of helper T-cell–oriented DNA vaccines.

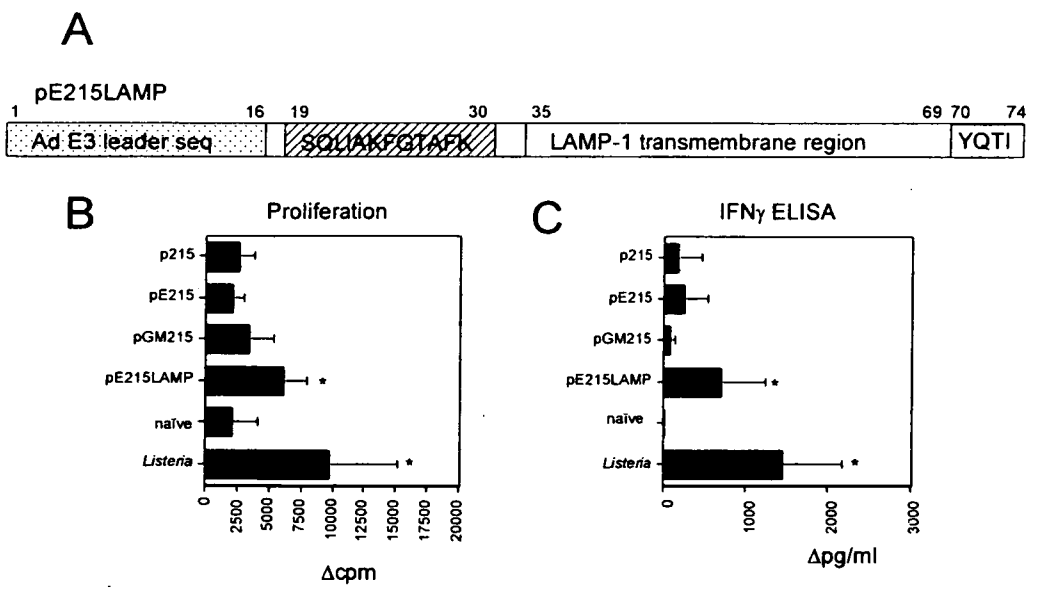

FIG. 7. Induction of LLO 215–226-specific Th by vaccination of plasmid for LLO 215–LAMP fusion molecule. (**A**) Schema of LLO 215–LAMP fusion molecule whose expression plasmid (pE215LAMP) was used for DNA vaccine. (**B**) LLO 215–226-specific splenocyte proliferation. C3H/He mice were immunized with the plasmid by gene gun bombardment four times. Immune spleen cells were harvested and cultured in vitro with LLO 215–226 peptide for 2 days and then [^{3}H] thymidine was added for the last 12 h. Asterisks indicate statistical significance ($P < 0.001$) compared with the value of naive mice. C. LLO 215–226-specific IFNγ production by immune spleen cells. The immune spleen cells were cultured with LLO 215–226 peptide for 5 days. The IFNγ concentration in the supernatants was qualified by sandwich enzyme-linked immunosorbent assay. Asterisks indicate statistical significance ($P < 0.005$) compared with the value of naive mice (modified figures of Uchiyama *et al.*, 2002).

IMPROVEMENT OF IMMUNIZATION REGIMEN: PRIME-BOOST METHOD FOR DNA IMMUNIZATION

In addition to the design of DNA vaccine plasmids, immunization protocols also need to be taken into consideration for enhancing immunological effects. Recent evaluation of DNA immunization indicates that DNA immunization alone has a limitation in terms of overall effects, and that the combination of different immunization methods gives better end results. As a pilot study, Li *et al.* (1993) reported that the combination of priming with recombinant influenza virus expressing the CTL epitope derived from *P. yoelii* circumsporozoite protein (PyCSP) and boosting with PyCSP-expressing vaccinia virus yielded more protection than immunizing with either PyCSP-expressing influenza virus or vaccinia virus alone, or the opposite order of prime-boost regimen (with vaccinia-PyCSP first and the recombinant influenza second). Similarly, a DNA heterologous prime-boost regimen, that is, primed with naked DNA vaccination and boosted with recombinant viral vectors such as vaccinia virus and adenovirus, has been shown to evoke superior levels of immunity (McShane, 2002; Ramshaw and Ramsay, 2000). Such a regimen has been evaluated in several infection models including malaria, HIV-1, tuberculosis, and herpes simplex virus (HSV) (Kent *et al.*, 1998; Schneider *et al.*, 1998; Sedegah *et al.*, 1998; Hanke *et al.*, 1999; Allen *et al.*, 2000; Sullivan *et al.*, 2000; McShane *et al.*, 2001). So far, the mechanisms of prime-boost vaccination are not clear. Even so, the relatively low-level, but persistent, expression of immunogenic proteins *in vivo* by naked DNA vaccines is suggested by Ramshaw and Ramsay (2000) to be important to prime immunological responses and to induce enhanced cellular immunity. Interestingly, Eo *et al.* (2001) reported that mucosal immunological responses were optimal when animals were primed with recombinant vaccinia virus vector and boosted with a naked DNA vaccine, which is an opposite approach compared with the regimen for systemic immunological responses.

SUMMARY

CTL and Th are key effectors in cell-mediated immunity. Here, we reviewed a variety of trials to induce specific T-cell subsets efficiently by DNA immunization. One of the excellent features of DNA vaccines is its extraordinary flexibility in designing the constructs by virtue of recombinant DNA technology. Various modifications of DNA vaccines have been examined and reported. It is important to develop excellent vaccines capable of inducing only particular favorable immune responses while preventing adverse responses by taking advantage of the flexibility in DNA vaccine design.

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Immunization with a gene encoding granulocyte-macrophage colony-stimulating factor inserted with a single helper T-cell epitope of an intracellular bacterium induces a specific T-cell subset and protective immunity

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Abstract

We evaluated here the effect of immunization with a gene encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) inserted with a helper T cell (Th) epitope, listeriolysin O (LLO) 215–226 derived from *Listeria monocytogenes* on induction of a specific Th by gene gun bombardment. Immunization of C3H/He mice with pGM215m plasmid encoding murine GM-CSF inserted with LLO 215–226 Th epitope gave the epitope-specific proliferative responses of $CD4^+$ T lymphocytes. In addition, specific interferon-γ production from the splenocytes was observed. Concomitantly, pGM215m-immunized mice showed significant protective immunity against lethal listerial challenge. These results suggest that immunization of a gene for GM-CSF inserted with a Th epitope is useful for eliciting a specific Th subset in vivo.
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Keywords: DNA immunization; GM-CSF; Th epitope

1. Introduction

Helper T cells (Th) play pivotal roles in many aspects of infection immunity, especially for modulating immune responses by producing special sets of cytokines. For protection against intracellular bacteria, activation of macrophages is indispensable and type 1-helper T cells (Th1) are important for the activation. The DNA vaccination method which induces only a particular Th population without production of antibodies may be advantageous as antibodies could, in some cases, give undesirable consequences [1]. Here, we evaluated the effect of immunization with a gene encoding murine granulocyte-macrophage colony-stimulating factor (GM-CSF) inserted with a single H2-E^k-restricted Th epitope [residues 215–226 of listeriolysin O (LLO)] derived from *Listeria monocytogenes* [2] by gene gun bombardment.

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2. Materials and methods

2.1. Animals

C3H/He mice (between 6 and 18 weeks of age; Japan SLC, Hamamatsu, Japan) were maintained at the Institute for Experimental Animals, Hamamatsu University School of Medicine. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

2.2. Plasmid construction

The eukaryotic expression vector, pCI (Promega, Madison, WI) was used as a backbone plasmid for construction of plasmids for DNA immunization. The oligonucleotides used for p215m plasmid were, 5′-CCCGGG ATG AGC CAG CTG ATC GCC AAG TTT GGC ACC GCC TTT AAG TAG CCCGGG-3′ and the opposite-strand oligonucleotide,

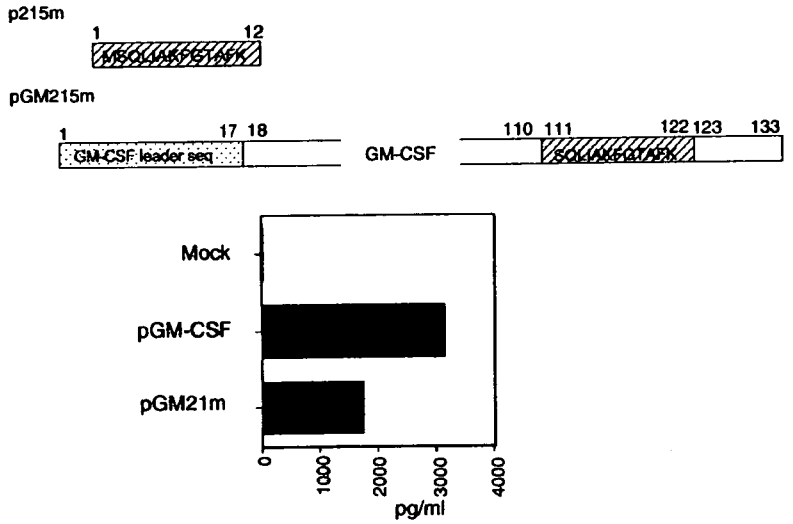

Fig. 1. (A) The schema of gene products deduced from the cDNA constructs prepared in this study (p215m and pGM215m). The hatched boxes indicate LLO 215–226 peptide and a dotted box indicate murine GM-CSF leader sequence. Amino acid numbers of each domain were shown above each schema. (B) Expression of GM-CSF inserted with LLO 215–226 peptide (GM215) in transfected cells. Supernatants of pGM-CSF- or pGM215m-transfected 293T cells were examined with ELISA specific to murine GM-CSF.

5′-CCCGGG CTA CTT AAA GGC GGT GCC AAA CTT GGC GAT CAG CTG GCT CAT CCCGGG-3′, which encode amino acid residues 215–226 of LLO, MSQLIAKFGTAFK and a termination codon. These oligonucleotides were annealed and inserted into the SmaI site of pCI (Fig. 1A). The codon usage of the oligonucleotide for LLO 215–226 peptide was optimized to that of *Mus musculus* [3]. pGM-CSF was constructed by inserting murine GM-CSF gene into the EcoRI/NotI sites of pCI. For pGM215m plasmid, a double-stranded oligonucleotide encoding LLO 215–226 was inserted in the unique EcoRV site of murine GM-CSF gene in pGM-CSF (Fig. 1A). The region is located in the region which should not affect the function of GM-CSF [4,5]. The nucleotide sequences of the resultant plasmids were confirmed by dideoxy sequencing by using ABI PRISM 310 Genetic Analyzer (Applied Biosystems; Foster City, CA).

2.3. Mice immunization

For DNA immunization with Helios gene gun system (Bio-Rad Laboratories, Hercules, CA), preparation of the cartridge of DNA-coated gold particle cartridge was followed to the manufacturer's instruction manual. Finally, 0.5 mg of gold particles was coated with 1 μg of plasmid DNA and the injection was carried out with 0.5 mg gold per shot twice. Mice were injected with 2 μg of plasmid DNA four times at 1-week intervals.

2.4. Enzyme-linked immunosorbent assay (ELISA) for GM-CSF

293T cells (human embryonal kidney cells) (approximately 5×10^6) were transfected with 2 μg of pGM-CSF or pGM215m using SuperFect Transfection Reagent (QIAGEN GmbH, Hilden, Germany). The supernatants were prepared 48 h after transfection and were assayed for GM-CSF using AN'ALYZA mouse GM-CSF Immunoassay Kit (G-T, Mineapolis, MN) according to the instruction manual.

2.5. Lymphocyte proliferation assay

Spleen cells (5×10^5 cells per well) from the immunized mice were incubated in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified 5% CO_2 atmosphere for 48 h at 37 °C in 96-well round-bottom tissue culture plates in the presence or absence of 1 μM of LLO 215–226 peptide. After 48 h in culture, de novo DNA synthesis was assessed by adding 0.5 μCi/well of [methyl-^{3}H] thymidine (10 Ci/mmol; ICN Biochemicals, Irvine, CA) for the last 12 h of culture. Triplicate cultures were harvested onto glass fiber filters, and the [methyl-^{3}H] thymidine incorporation was determined by counting the radioactivity (cpm) using a liquid scintillation counter.

2.6. ELISA for IFN-γ

Spleen cells were harvested from the immunized mice. Recovered cells were plated in 24-well plates at 2×10^6 cells/well in the presence or absence of 1 μM of LLO 215–226 peptide for 5 days. Concentration of IFN-γ in the culture supernatants was determined by a sandwich ELISA as described in our previous report [6].

2.7. Intracellular IFN-γ staining

The number of LLO 215–226-specific $CD4^+$ T-cell subset was examined by intracellular IFN-γ staining. Spleen cells