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Induction of $CD8^+$ Cytotoxic T Lymphocytes with MHC Class I Restriction by a Soluble Truncated Oncoprotein

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$CD8^+$ cytotoxic T lymphocytes (CTLs), which play a major role in the immunological defense against cancer, recognize endogenously produced peptides in the context of MHC class I molecules. We investigated how to induce $CD8^+$ CTL responses against the HER2/neu/c-erbB2 (HER2) oncoprotein often overexpressed in a wide range of human adenocarcinomas. The immunization of BALB/c mice with a syngeneic cell line transduced with HER2 cDNA led to a successful induction of $CD8^+$ CTLs which specifically destroyed HER2-expressing tumor cells. The CTLs recognized the HER2-derived peptide 1 (TYLPTNASL, pos. 63rd-71st amino acid) in the context of MHC class I K^d. The immunization of mice with a truncated HER2 oncoprotein containing 144 amino acids of HER2 (N terminus to 144th amino acid) failed to elicit measurable CTL activity for HER2-expressing target cells. We reconstituted the truncated HER2 protein into a mannan-coated liposome, and complexed the protein with a cholesterol-bearing mannan polysaccharide respectively. Both of these complexes were capable of inducing killer cells specific for HER2-expressing cells in murine model after immunization. These killer cells are K^d restricted $CD8^+$ CTLs which recognize peptide 1. The cholesterol-bearing mannan polysaccharide facilitated the induction of specific $CD8^+$ CTLs by an exogenous HER2 oncoprotein, and may therefore be useful in the development of cancer vaccines.

Key words : $CD8^+$CTL, Exogenous protein, Polysaccharides, Mannan, Tumor vaccine, Liposome

Introduction

A variety of immunocompetent cells are involved in the host immune responses against cancer cells. Among them, cytotoxic T lymphocytes (CTLs) have been considered a major effector cell population[1-3], and display immunologically specific cytolytic activity against cancer cells in *in vitro* CTL assays with ^{51}Cr-labeled target cells. Most CTLs express a $CD8^+$ phenotype and destroy target cells in a major histocompatibility complex (MHC) class I antigen restricted manner. MHC class I molecules present antigen peptides which are primarily derived from endogenously produced protein molecules[4-6]. It is therefore speculated that $CD8^+$ T cells must be directly sensitized by cancer cells or cells producing the cognate antigen peptides in order to become specific killer cells.

In certain types of cancer, such as melanoma and adenocarcinomas, antigen peptides recognized by CTLs were identified[1,7,8]. These findings prompted investigators to attempt to develop cancer vaccines which can induce specific anti-cancer CTL activity. However, experimental trials to vaccinate hosts with 8 to 10 mer antigen peptides recognized by CTLs generally resulted in weak or no priming activity[9-11]. Although the use of recombinant proteins consisting of antigen peptide sequences seems very promising, the failure to induce specific CTL activity by immunizing hosts with such protein molecules has been repeatedly experienced[12-16]. It is therefore important to develop a novel approach to induce MHC class I restricted CTL activity by the use of recombinant proteins. Noguchi et al[13]. have previously reported to use a recombinant protein reconstituted into a mannan coated liposome. In a human T lymphotropic virus type I (HTLV-I) induced rat lymphoma system, the authors were able to prime host animals by recombinant truncated gag-env proteins reconstituted into liposomes for generating MHC class I restricted CTLs. The CTLs specifically lysed HTLV-I^+, but not HTLV-I^- tumor cells. This initial success in utilizing polysaccharide-coated liposomes encouraged us to develop more advanced derivatives useful for the induction of cancer-specific $CD8^+$ CTLs.

We report here the first analysis to utilize a cholesterol-bearing mannan polysaccharide complexed with recombinant oncogene products of HER2/neu/c-erbB2 (HER2) for the induction of HER2- specific $CD8^+$ CTLs.

Materials and Methods

Mice. For all experiments, 6-8 week-old female BALB/c mice were used, which were purchased from Shizuoka Animal Laboratory Center (Shizuoka, Japan) or bred at

the Laboratory Animal Centers of Nagasaki University School of Medicine and Mie University School of Medicine.

Tumor cell lines. CMS7, CMS8 and CMS17 are 3-methylcholanthrene induced sarcoma cell lines of BALB/c origin[17]. P1HTR is a mastocytoma cell line of DBA/2 origin.

Transfection. Full-length HER2 cDNA was inserted into the pBCMGSneo vector to prepare expression plasmids. CMS7, CMS8 and CMS17 were transfected by expression plasmids using the electroporation method. Positive clones were selected by G418 (GIBCO, Grand Island, NY) at a concentration of 250 μg/ml. After this selection, the expression of HER2 on transfected tumor cells designated CMS7HE, CMS8HE and CMS17HE was checked by FACS analysis (Becton Dickinson, Mountain View, CA), using anti-HER2 mAb (rhu 4D5).

Monoclonal antibodies. Anti-HER2 mAb (rhu 4D5) was kindly provided by Mitsubishi Chemical Cooperation, Yokohama, Japan. Anti-L3T4 (CD4) mAb, produced by hybridoma GK1.5 was provided by Dr. F. Fitch (Chicago University, Chicago, IL). Anti-Lyt-2.2 (CD8) mAb, produced by hybridoma 19/178, was provided by Dr. U. Hammerling (Memorial Sloan-Kettering Cancer Center, New York, NY). Anti-H-$2K^d$ mAb, produced by hybridoma 20-8-4, and anti-H-$2L^d$ mAb, produced by hybridoma 30-5-7, were provided by Dr. D. Sachs (NIH, Bethesda, MD). Anti-H-$2D^d$ mAb, produced by hybridoma T17/633, was provided by Dr. N. Tada (Tokai University School of Medicine, Isehara, Japan).

Preparation of cholesterol-bearing mannan suspension. Cholesterol-bearing mannan (CHM) was synthesized according to the method previously reported[18]. Mannan (MW = 85000) was substituted by 2.3 cholesterol moieties per 100 mannose of mannan. The cholesterol-bearing polysaccharide was dissolved in DMSO and dialyzed against PBS (150 mM, pH 7.9). After dialysis, the suspension was sonicated using a probe type sonifier (UR-200P, Tomy, Tokyo) at 40 W for 10 min. The obtained suspension was filtered through three types of membrane filter (Super Acrodisc 25, pore size : 1.2 μm, 0.45 μm. 0.2 μm, Gelman Science) to remove dust. Finally, clear suspensions were obtained[18,19]. The CHM and its complex with HER2 protein (CHM-HER2) were prepared and kindly provided by Drs. K. Akiyoshi and J. Sunamoto, Kyoto University Graduate School of Engineering, Kyoto, Japan.

Preparation of the complex of a truncated HER2 protein and cholesterol-bearing mannan. The HER2 derived truncated protein consisting of the amino residues 1 to 144 was kindly provided by Dr. H. Nakamura, Mitsubishi Chemical Corporation, Yokohama. The HER2 protein (2.0 mg/ml) was dissolved in 6M urea and mixed with 2.1 ml of the suspension of a cholesterol-bearing mannan polysaccharide (5.7 mg/ml) at room temperature, resulting in the formation of a CHM-HER2 complex (protein : 0.25 mg/ml)[20].

Preparation of liposomes. The HER2-derived truncated protein was reconstituted into a CHM-coated liposome (CHM-lipo-HER2) and a conventional liposome, as described elsewhere[13]. The liposomes and their reconstitution into HER2 protein were also prepared and kindly provided by Drs. K. Akiyoshi and J. Sunamoto, Kyoto University.

Synthetic peptides. Peptide 1 (TYLPTNASL), peptide 2 (CYGLGMEHL), peptide 3 (EYVNARHCL) and peptide 5 (PYVSRLLGI) were derived from HER2 protein to correspond to the H-$2K^d$ binding motif, and were synthesized and purified at Chiron Mimotopes (Australia).

Immunization. 1. BALB/c mice were immunized subcutaneously twice at a one-week interval with mitomycin-C (MMC)-treated $1x10^6$ CMS17HE.
2. BALB/c mice were immunized subcutaneously twice at a one-week interval with CHM-lipo-HER2 complex or CHM-HER2 complex (HER2 protein 20 μg per immunization).

Generation of CTL. One week after the second immunization with CMS17HE, CHM-lipo-HER2 or CHM-HER2, the mice were sacrificed and their spleen cells were obtained. Cells from these immunized mice were suspended at a concentration of $4x10^6$/ml in RPMI 1640 with 20% FCS, $5x10^{-5}$M 2-ME, penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (0.2 mg/ml). Then, $4x10^7$ spleen cells were cocultured with $4x10^6$ MMC-treated CMS17HE tumor cells in upright 50 ml tissue culture flasks in a total volume of 20 ml of medium at 37℃ in 5% CO_2 for 1 week. Cytotoxic activity was examined by the ^{51}Cr-release assay.

^{51}Cr-release CTL assay. The assay was performed as described elsewhere[21].

Inhibition of in vitro cytotoxity by mAb. 50 μl volumes of serially diluted mAb were incubated with a mixture of $4x10^5$ immunized spleen cells suspended in 50 μl of 10% FCS RPMI1640 and $1x10^4$ ^{51}Cr-labeled target cells suspended in 100 μl of 10% FCS RPMI1640 for 12 hr at 37℃.

Peptide pulsing. $1x10^6$ P1HTR cells were washed by plain RPMI 1640 and then resuspended in 500 μl of plain RPMI 1640 in a 14 ml round bottom tube (Falcon). 10 μl of peptide solution (1 mg/ml in PBS) was added, followed by incubation for 15 min at room temperature. Then, 500 μl of RPMI1640 with 20% FCS was added followed by incubation for 45 min at room temperature, with the tube being tapped every 15 min. Another round of incubation at 37℃

for 1 hr followed, the solution being tapped every 15 min. Finally, the suspension was washed twice by plain RPMI1640. Thereafter, the pulsed cells were used as target for CTL in a 4-hr ^{51}Cr-release assay.

Results

Expression of HER2 in transfectants.

The expression plasmids for HER2 were transduced into the 3-methylcholanthrene induced sarcoma lines CMS7, CMS8 and CMS17, all of BALB/c origin. Their expression was examined by flowcytometry utilizing a mAb specific for the extracellular domain of HER2 protein. A representative result of a flowcytometry analysis is shown in Fig. 1. Transfectants of the HER2 cDNA plasmid, but not those of a control neomycin resistance gene plasmid, expressed HER2 molecules on their cell surface .

Generation of CTL specific for HER2 expressing cells.

BALB/c mice were immunized two times at a one-week interval by subcutaneous injections of 1x10^6 CMS17HE (CMS17 transfected with HER2 cDNA). CMS17HE was pretreated by MMC before injection. One week after the second immunization, spleen cells were taken out from immunized mice and sensitized in vitro with MMC treated CMS17HE. The cells were kept in culture for several weeks by repeating the sensitization at a weekly basis with CMS17HE. Several CTL lines specific for HER2 expressing target cells were generated. An example is shown in Fig. 2. These CTLs were CD8$^+$ T cells with the restriction of a MHC class I K^d molecule, as shown in Fig. 3.

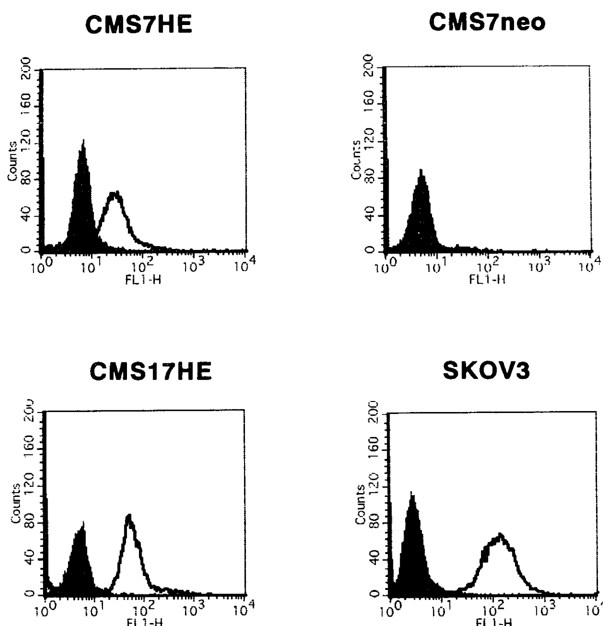

Fig. 1. Flowcytometry examination of HER2 expression was performed using the mAb rhu4D5. The HER2 transfectants CMS7HE (upper left) and CMS17HE (lower left) showed HER2 expression (unfilled curves), but CMS7neo, transfected with the neomycin resistance gene alone, did not (upper right). SKOV3 with known strong HER2 expression (lower right) served as a positive control. The filled curves represent cells not treated with the 1st antibody (rhu4D5).

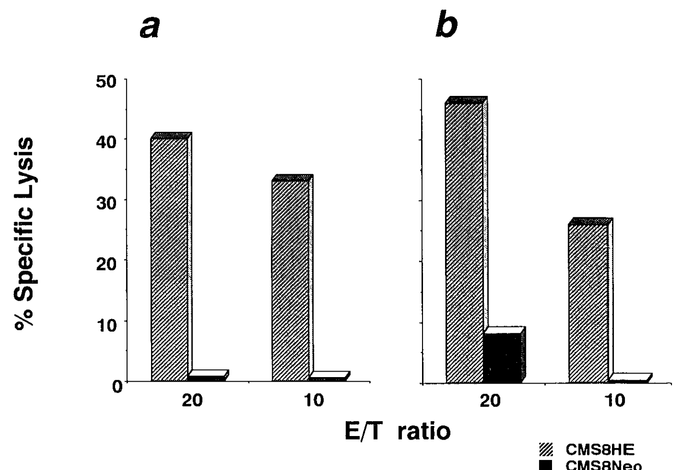

Fig. 2. ^{51}Cr release cytotoxicity assays were performed with 2 CTL lines derived from BALB/c mice (*a* & *b*) immunized in vivo twice at a weekly basis, and also sensitized in vitro with CMS17HE tumor cells. The target cells are HER2 transfected CMS8HE tumor cells (▨). CMS8neo tumor cells (■) without HER2 expression served as a control.

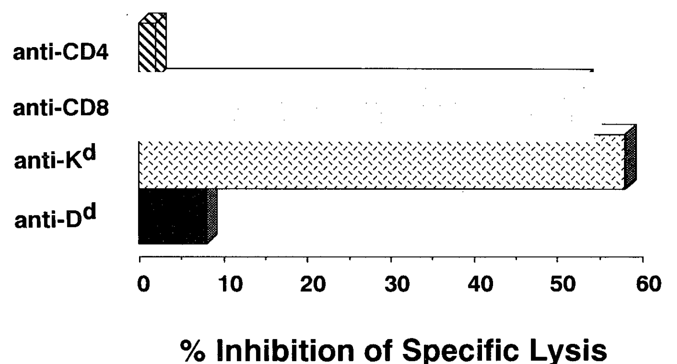

Fig. 3. The characteristics of the killer cells induced by CMS17HE tumor cells were examined in vitro by inhibiting their activity with the mAb as specified in the figure. The percentage of inhibition was calculated as follows :% inhibition = (1-% specific lysis with mAb treatment/% specific lysis without mAb treatment) x 100%. Anti-CD8 and anti-K^d mAbs demonstrated a significantly high inhibition of the cytotoxicity of these killer cells.

CTL recognize HER2 derived peptides.

Since the motif of peptides binding to K^d molecules has already been identified, we prepared synthetic peptides matching the motif based on the amino acid sequences of

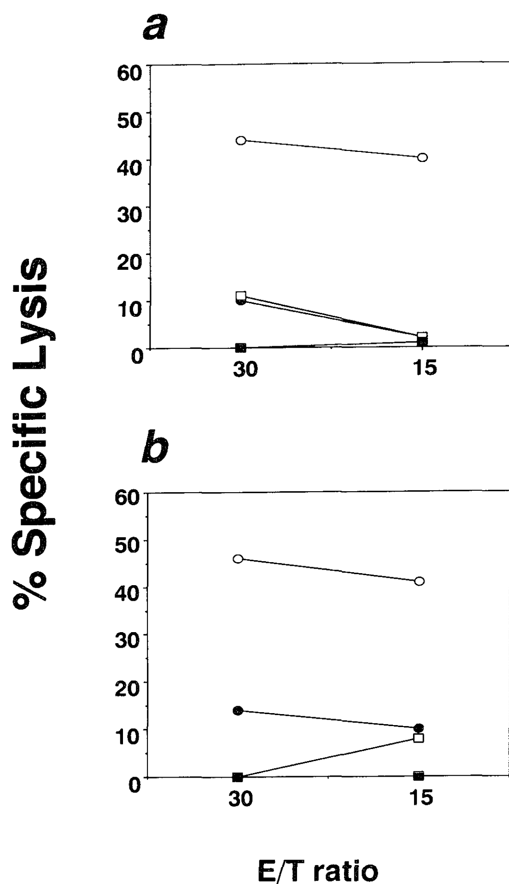

Fig. 4. ^{51}Cr release cytotoxicity assays were performed with 2 CTL lines derived from BALB/c mice (*a* & *b*) immunized with CMS17HE tumor cells. Immunized spleen cells were repeatedly sensitized in vitro with CMS17HE tumor cells, and then tested for the cytotoxicity against P1HTR cells (DBA/2, H-2^d) which were pulsed with HER2 derived peptide 1 (open circles), peptide 2 (filled circles), peptide 5 (open squares), or nothing (filled squares). CTLs specifically recognized peptide 1 containing the characteristic anchor motif for K^d.

HER2 as described in the Materials and Methods section. The reactivity of CTL lines specific for HER2 expressing cells was examined with P1HTR target cells, an HER2 negative and K^d positive mastocytoma line of DBA/2 origin, pulsed with synthetic peptides. Several CTL lines showed strong CTL activity for the P1HTR target cells pulsed with peptide 1 (TYLPTNASL, pos. 63rd-71st amino acid) derived from HER2 (Fig. 4).

Generation of HER2 specific CTL by truncated HER2 protein complexed with CHM or reconstituted into CHM-liposome.

We prepared a truncated protein which consists of the 144 N-terminal amino acid residues of HER2. The truncated HER2 molecules were complexed with a hydrophobic mannan polysaccharide (CHM) as described in Materials and Methods. They were also reconstituted into mannan coated liposome (CHM-liposome) as previously described[13].

BALB/c mice were subcutaneously injected two times at a one-week interval with CHM-HER2 or CHM-lipo-HER2. After the second injection, spleen cells were taken out and sensitized in vitro with CMS17HE. Lymphocytes derived from mice injected with either CHM-HER2 or CHM-lipo-HER2 showed specific cytotoxity against HER2 expressing target cells. The results of a representative experiment are

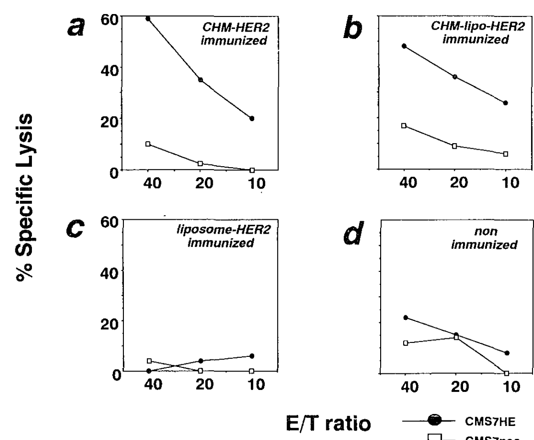

Fig. 5. The killer cell activity of spleen cells derived from mice immunized respectively with *a*. CHM-HER2, *b*. CHM-lipo-HER2, or *c*. liposome (non mannan coated)-HER2 were compared in CTL assays. *d*. The spleen cells from non immunized mice served as a control. The target cells are specified in the graph inlet. BALB/c mice were immunized with *a*, *b* or *c* twice at a one-week interval, and the spleen cells were sensitized in vitro with MMC-treated CMS17HE before the CTL assays.

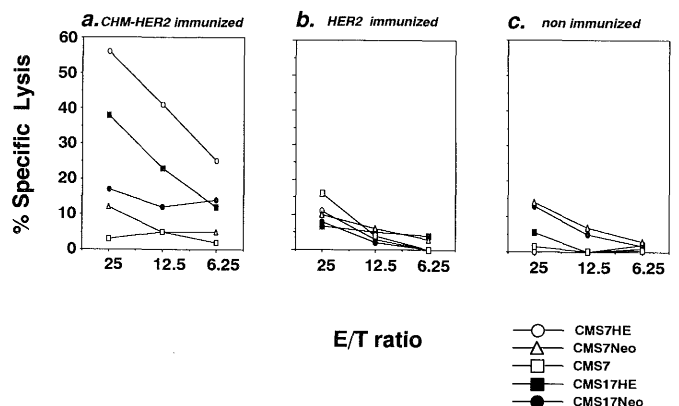

Fig. 6. The killer cell activity derived from mice immunized with *a*. CHM-HER2, or *b*. HER2 oncoprotein alone was compared in CTL assays. *c*. The spleen cells from non immunized mice served as a control. The target cells are specified in the graph inlet. BALB/c mice were immunized with *a* or *b* twice at a one-week interval, and the spleen cells were sensitized in vitro with mitomycin-C treated CMS17HE before CTL assays.

shown in Fig. 5. To test whether the protein alone can elicit killer activity, another experiment was performed by immunizing mice with CHM-HER2 or HER2 alone. As shown in Fig. 6, lymphocytes derived from mice immunized with CHM-HER2 but not from those immunized with HER2 alone showed specific lysis of HER2 expressing target cells.

Killer cells induced with CHM-HER2 specifically recognize an HER2 derived peptide in the context of MHC class I.

Characteristics of the CHM-HER2 induced killer cells were examined by blocking the killer cell activity with monoclonal antibodies for cell surface molecules. The killer cell activity was blocked by antibodies for CD8 and K^d, but not for CD4, D^d or L^d (Fig. 7), which indicates that the killer cells are $CD8^+$ CTLs with MHC class I K^d restriction. Since the sequence of the truncated HER2 protein contains the peptide 1 with the K^d binding motif, we examined whether the CTLs were directed against peptide 1. As shown in Fig. 8, they specifically destroyed peptide 1 pulsed P1HTR cells, but not target cells pulsed with other peptides bearing the K^d binding motif.

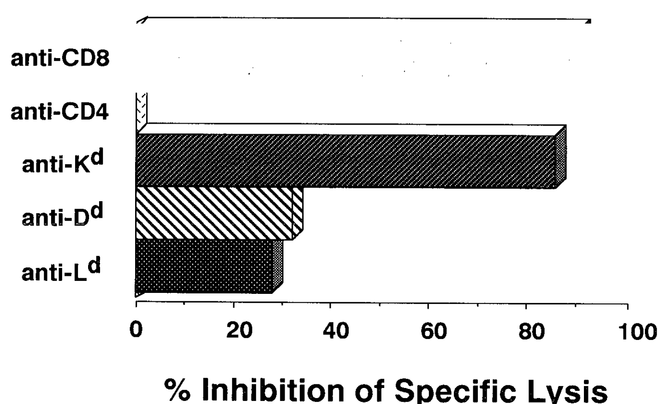

Fig. 7. Characteristics of killer cells induced by CHM-HER2 were examined in vitro by inhibiting the killer cell activity with the mAbs specified in the figure. The CTL assay was performed with such treated killer cells. The percentage of inhibition was calculated as described in Fig. 3.

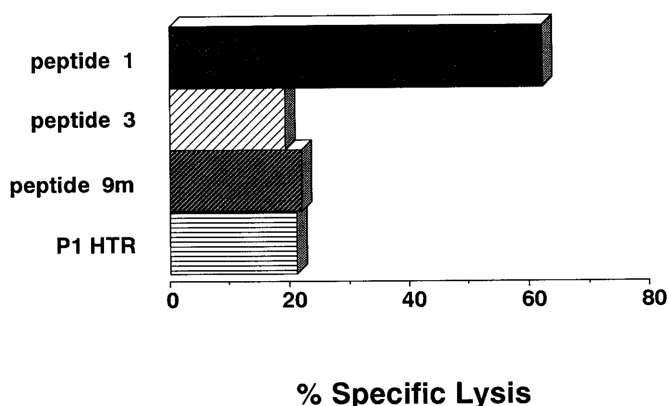

Fig. 8. A ^{51}Cr-release assay was performed with CTLs induced with CHM-HER2 to test the specific cytotoxicity for HER2 derived peptide 1, peptide 3, or the irrelevant ERK2 derived peptide 9m, all of which were pulsed to P1HTR cells (DBA/2, H-2^d). Unpulsed P1HTR served as a control. The results are similar to those shown in Fig. 4.

Discussion

For the analysis of T cell mediated immunity directed against products of a proto-oncogene, HER2, we prepared a panel of cell lines transduced with the whole cDNA of HER2. Each of these three 3-methylcholanthrene induced sarcomas of BALB/c origin expressed the transduced genes. This panel of transfectants, in addition to transfectants of the neomycin resistance gene as well as parental cells, enabled us to define the specificity of the elicited T cell mediated immunity. The appropriate immunization of syngeneic BALB/c mice with HER2 expressing cells enabled the generation of CTLs directed for HER2 gene products. One of the dominant antigen epitopes recognized by CTLs with K^d restriction was presented by peptide 1, consisting of the 63rd to the 71st amino acid residues of HER2. A comparison of the peptide sequence of HER2 with that of the murine homologue revealed that they differ in only one amino acid. The further analysis demonstrated that the corresponding peptide of the murine homologue could also induce specific CTLs, supporting the immunogenicity of the peptide in murine hosts (Nagata et al: Peptides derived from a wild type murine proto-oncogene c-erbB-2/HER2/neu can induce CTL and tumor suppression in syngeneic hosts. J Immunol in press). These observations, together with several reports on HER2 derived peptides recognized by HLA-A2 restricted CTLs[8,22,23] clearly indicate the potential usefulness of HER2 gene products for the induction of cancer-directed T cell immune responses.

Studies of tumor immunology have emphasized the role of $CD8^+$ CTLs as the major effector cells in the immunological defense against cancer. $CD8^+$ T cells recognize 8-10mer antigen peptides derived from endogenously produced protein molecules and presented by MHC class I molecules. In contrast, the exogenous proteins elicit $CD4^+$ T cell immune response through the MHC class II pathway[16]. Since antigen peptides with cancer specificity have been identified, it appears possible to develop efficient vaccination technologies to utilize either antigen peptides or recombinant proteins containing antigen peptide sequences. In fact, these would be more desirable as vaccines than tumor cells or transfectants of DNA sequences coding for peptides, from both of which potentially aberrant effects have never been fully excluded. As

shown in the present analysis, a cholesterol-bearing mannan polysaccharide functions as appropriate carrier of antigen proteins comparable with mannan liposomes, whose usefulness was previously reported[12]. The cholesterol-bearing polysaccharide used in the present study could turn out to be even more useful since they present far less potential to cause side effects such as microembolisms. In addition, their preparation is much less cumbersome when compared with liposomes[18-20].

As to how cholesterol-bearing polysaccharides help proteins to enter the MHC class I pathway, there are several conceivable explanations. The coating by cholesterol-bearing polysaccharides may protect proteins by maintaining their molecular stability, and may prevent the antigen proteins from being cleaved by enzymes in the endosome, and thus entering the MHC class II pathway. Since CHM has a lipophilic cholesterol moiety, this complex may help the encoated proteins to pass through the membrane of an endosome into the cytosol or even directly enter the cytosol through the cell membrane due to its lipid bilayer[18,19,24]. Alternatively, because of the lipophilic character of cholesterol-bearing polysaccharides, CHM may allow antigens to directly associate with MHC class I molecules on the lipid bilayer membrane of antigen presenting cells[24] after the cleavage of proteins for the preparation of antigenic peptides. CHM may elicit cytokines such as Interleukin(IL)-12 and Interferon(IFN)-γ, and drive on the maturation and proliferation of type 1 T helper cells, by which some cytokines as IL-2 and IFN-γ are subsequently produced and serve as the second signal for the maturation of naive precursor T cells towards functional CD8$^+$ CTLs[25].

The investigation of these possibilities would be indispensable towards determining the usefulness of CHM-HER2 and for the development of more advanced derivatives. It is critical to determine how effective CHM-HER2 is for the in vivo protection against and the treatment of HER2-expressing tumors. These studies are ongoing in our laboratory.

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