Liposome oligomannose-coated with neoglycolipid, a new candidate for a safe adjuvant for induction of $CD8^+$ cytotoxic T lymphocytes

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Abstract The cytotoxic T lymphocyte (CTL) response has recently been shown to play a role in protection against human immunodeficiency virus (HIV) and it is therefore thought that a vaccine against HIV must be able to elicit a CTL response. The development of a safe, effective adjuvant is very important because alum, the only adjuvant available for use in humans at present, can barely induce a response of this type. We demonstrate here that liposomes that contain an immunodominant peptide (15 amino acids) of the envelope glycoprotein gp120 of HIV-1 and that are coated with mannopentaose-dipalmitoylphosphatidylethanolamine conjugate induce a major histocompatibility complex class I-restricted $CD8^+$ CTL response in mice with a single subcutaneous immunization, whereas non-coated liposomes do not. Since no damage to the skin at the injection site was caused by the liposomes, and since the oligomannose-coated liposomes consist of innocuous materials ubiquitously distributed throughout the human body, they may be highly suitable for use as a safe adjuvant in vaccines inducing a CTL response against HIV.

Key words: Neoglycolipid; Vaccine; Adjuvant; Cellular immunity; Cytotoxic T lymphocyte; Human immunodeficiency virus

1. Introduction

Cell-mediated immune response such as the cytotoxic T lymphocyte (CTL) response is deeply involved in protective immunity against infection by human immunodeficiency virus (HIV) [1]. In HIV-1 infection, in addition to the production of antibodies, CTL responses may be detected that are specific to epitopes in the envelope glycoprotein, the gag proteins, and other HIV proteins including reverse transcriptase [2]. Among people infected with HIV, long-term non-progressors show an especially strong CTL response against HIV, suggesting that CTL may be able to control HIV infection and progression to AIDS [3,4]. Thus, elicitation of CTL response is considered to be required in a vaccine against HIV [5]. It is well-known that HIV proteins and their epitope peptides that induce a CTL response against HIV do not induce such responses unless they are administered together with an adjuvant. Although various adjuvants that induce cellular immunity have been found, they are used only in animals because of their toxicity to humans. Alum adjuvant, which is made of aluminum hydroxide or aluminum phosphate and which is the only adjuvant available for use in humans at present, cannot induce a response of this type. Therefore, the development of an adjuvant that is safe for humans and that can induce a CTL response is very important.

The surfaces of various infectious agents such as viruses [6], bacteria [7], fungi [8], and protozoa [9] are coated with carbohydrate moieties rich in mannose residues. Antigen-presenting cells such as macrophages and dendritic cells have been shown to express mannose receptors on the cell surface [10–15]. In the present study, we prepared oligomannose-coated liposomes using a neoglycolipid constructed with mannopentaose and dipalmitoylphosphatidylethanolamine (which are ubiquitously found in the human body) as a new candidate for a safe adjuvant and examined whether the liposomes encapsulating the epitope peptides of the HIV envelope glycoprotein gp120 perform an adjuvant activity to induce an epitope-specific CTL response.

2. Materials and methods

2.1. Materials

Synthetic peptides 18IIIB (RIQRGPGRAFVTIGK) and 18I10 (RGPGRAFVTI) were obtained from Iwaki Co. (Chiba, Japan). Dipalmitoylphosphatidylcholine and mannopentaose with a structure of Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man were purchased from Funakoshi Co. (Tokyo, Japan). Dipalmitoylphosphatidylethanolamine and cholesterol were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Anti-CD8 (anti-Ly-2.2) monoclonal antibody and rabbit complement (Low-Tox-M Rabbit Complement) were obtained from Cederlane Laboratories Ltd. (Ontario, Canada).

The neoglycolipid-containing oligomannose was prepared by conjugation of mannopentaose and dipalmitoylphosphatidylethanolamine using a method described previously [16,17].

2.2. Preparation of oligomannose-coated liposomes

Liposomes (multilamellar vesicles) were prepared as follows: 750 μl of a chloroform-methanol (2:1, v/v) solution containing 5 μmol of dipalmitoylphosphatidylcholine and 2.5 μmol of cholesterol was placed in a conical flask and rotary evaporated to prepare a lipid film. 250 μl of PBS or PBS containing 1.25 mg of synthetic peptide was added to the dried lipid film and multilamellar vesicles were then prepared by intense vortex dispersion. The amount of liposomes thus obtained was measured as the amount of dipalmitoylphosphatidylcholine. Liposomes were coated with oligomannose by incubation with the neoglycolipid at 12°C for 3 days. The coating of liposomes with the neoglycolipid was confirmed by their agglutination by concanavalin A and by composition analysis of the coated liposomes.

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Abbreviations: CTL, cytotoxic T lymphocyte; HIV, human immunodeficiency virus; Man, mannose; PBS, phosphate-buffered saline

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2.3. Immunization of mice

BALB/c mice were subcutaneously immunized for CTL assay once on both sides of the dorsal skin near the base of the tail with the liposomes, which contained 200 μg of dipalmitoylphosphatidylcholine and 0 or 3 μg of peptide, and which were either coated or not coated with 6 μg of the neoglycolipid. BALB/c mice given PBS were used as non-immunized mice. Separately, BALB/c mice were intravenously immunized once with the same amount of the oligomannose-coated liposomes containing the peptide.

In order to examine production of antibody against oligomannose, 30 μg of the neoglycolipid containing the mannopentaose was subcutaneously injected to BALB/c mice three times at intervals of 3 weeks. Mice given PBS were used as non-immunized mice. One week after the last injection, sera were analyzed for antibody production by enzyme immunoassay using the neoglycolipid as antigen.

2.4. CTL assay

Four to six weeks after immunization, spleen cells (5×10^6 cells/ml of RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol) were re-stimulated in vitro with 0.3 μM peptide 18IIIB. After 6 days of culture, the cytolytic activity of the re-stimulated cells was measured using a conventional 4-h assay with target cells (1×10^4 cells per well) at the indicated effector:target cell ratios. Syngeneic P815 cells pulsed with or without 1 μM of 10-mer peptide 18I10 were labeled with $Na^{51}CrO_4$ and used as target cells. The percent specific ^{51}Cr release was calculated as: $100\times$(experimental release−spontaneous release)/(total release−spontaneous release). Total release was determined from the radioactivity in the supernatants of cells that were lysed by addition of 5% Triton X-100. Results are expressed as % specific ^{51}Cr release.

3. Results and discussion

The synthetic peptide of the CTL epitope (18IIIB; RIQRGPGRAFVTIGK) from the envelope glycoprotein gp120 of HIV-1 [18,19] was incorporated into liposomes, which were then coated with oligomannose using a neoglycolipid constructed with Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man and dipalmitoylphosphatidylethanolamine. BALB/c mice were subcutaneously immunized with the liposomes and induction of CTLs was examined. As shown in Fig. 1, CTLs that specifically kill target cells pulsed with a synthetic peptide of the CTL epitope (18I10; RGPGRAFVTI) of the envelope glycoprotein gp120 of HIV-1 [20] were obtained from mice subcutaneously immunized with a single dose of oligomannose-coated liposomes containing the 15-mer peptide 18IIIB. However, none were obtained from mice inoculated with non-coated liposomes containing the peptide, from oligomannose-coated or non-coated liposomes containing no peptide or from non-immunized mice. No microabscesses and no inflammation was observed at skin sites where the coated or non-coated liposomes had been injected. CTLs specific for epitope-pulsed target cells were also obtained even from mice immunized with oligomannose-coated liposomes containing 40 μg of dipalmitoylphosphatidylcholine, 1.2 μg of the neoglycolipid, and 0.6 μg of the peptide (which corresponds to one-fifth of the liposomes used in the present study), although the lytic activity of the CTL was slightly lower than that obtained in the present study (data not shown). These results indicate that liposomes that are oligomannose-coated with the neoglycolipid perform an adjuvant activity to induce epitope-specific CTL, but have no toxicity.

In order to examine whether differences in route of immunization would result in different effects, the same amount of the oligomannose-coated liposomes containing the peptide was injected intravenously. The lytic activity of the CTL induced by intravenous injection was somewhat weaker than that of cells induced by subcutaneous injection (Fig. 1). This weaker CTL response may be due to degradation of the liposomes in the circulation, resulting in a decrease in the population of CTLs induced by immunization. In any case, the injection route is important and its significance should be further examined, since liposomes must be delivered effectively to appropriate regions (or cells) before degradation occurs in vivo.

We then examined the phenotype of the CTLs primed with the oligomannose-coated liposomes containing the peptide. Effector cells from BALB/c mice immunized with oligoman-

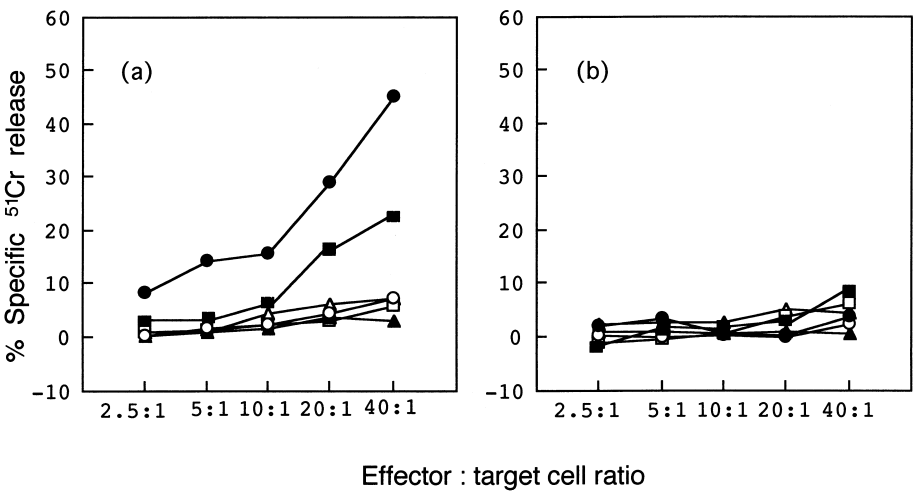

Fig. 1. Induction of epitope peptide-specific CTL activity by immunization with oligomannose-coated liposomes containing 15-mer peptide 18IIIB. Syngeneic target P815 cells (mastocytoma expressing H-2^d) were (a) pulsed with 10-mer peptide 18I10 or (b) untreated. Lytic activity was measured using spleen cells from BALB/c (H-2^d) mice subcutaneously immunized with (1) oligomannose-coated liposomes containing 18IIIB (filled circles), (2) non-coated liposomes containing 18IIIB (open circles), (3) oligomannose-coated liposomes containing no peptide (filled triangles), or (4) non-coated liposomes containing no peptide (open triangles); using spleen cells from BALB/c mice intravenously immunized with the oligomannose-coated liposomes containing 18IIIB (filled squares); or using spleen cells from non-immunized BALB/c mice (open squares).

nose-coated liposomes containing peptide 18IIIB were prepared as described in Section 2. The cells were then pretreated with anti-CD8 monoclonal antibody and rabbit complement or with the complement alone. As shown in Fig. 2, the lytic activity of the induced CTL was inhibited by pretreatment of the cells with a mixture of anti-CD8 monoclonal antibody and rabbit complement, but not with complement alone. This result indicates that lysis of the target cells was caused by $CD8^+$ CTL.

We also examined whether the lytic activity of the induced CTL was restricted by major histocompatibility complex (MHC) class I molecules. The lytic activity of the CTL induced in BALB/c (H-2^d) mice was examined not only when using P815 cells expressing H-2^d as target cells but also when using EL4 cells and BW5147 cells expressing H-2^b and H-2^k, respectively. As shown in Fig. 3, the induced CTLs were able to kill peptide 18I10-pulsed P815 cells but unable to kill the peptide-pulsed EL4 and BW5147 cells. In addition, the CTLs could lyse not only the P815 cells but also fibroblast-like cells (RGB3T3-1) that express H-2^d (data not shown). These results indicate that the lytic activity of the induced CTL was MHC class I-restricted. From the results so far described, it was demonstrated that a single immunization with a synthetic peptide encapsulated into the oligomannose-coated liposomes can induce epitope-specific, MHC class I-restricted, and $CD8^+$ CTL.

The mechanism by which the oligomannose-coated liposomes containing a soluble form of peptide induced the CTLs is unknown. It is conceivable that a mechanism for recognizing mannose-rich oligosaccharide structures on the surface of various infectious agents may have arisen as part of a defense system utilizing activation of the immune system. Recent studies have suggested the existence of an interaction between mannose and mannose receptors on antigen-presenting cells, such as macrophages and dendritic cells, which are involved in cellular immunity [10–15]. It was shown that mannose receptors bind strongly to oligosaccharides with mannose at their non-reducing termini. These findings are consistent with our preliminary observations that, among the various oligosaccharides used to coat liposomes, only the oligomannose type with non-reducing terminal mannose residues had adjuvant activity that induced the cellular immune response. Therefore, specific delivery of the epitope peptide to the antigen-presenting cells would have been performed by the oligomannose residues of the neoglycolipid on the surface of the liposomes. The priming of CTLs in mice immunized with oligomannose-coated liposomes containing epitope peptide could be ascribed to the interaction between mannose residues on the surface of the liposomes and mannose receptors on the antigen-presenting cells. In addition, oligomannose residues may perform some other activity, such as stimulation of IL-12 release, which would result in the activation of T-lymphocytes.

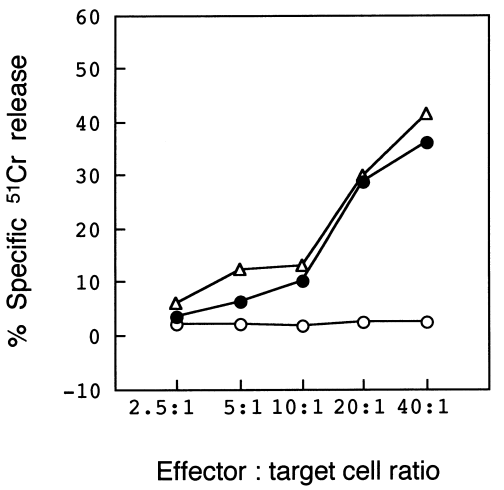

Fig. 2. Oligomannose-coated liposome adjuvant induces $CD8^+$ CTL. Effector cells from BALB/c mice immunized with oligomannose-coated liposomes containing peptide 18IIIB were prepared as described in Section 2. The cells were then pretreated with anti-CD8 monoclonal antibody and rabbit complement (open circles) or with complement alone (open triangles), or left untreated (filled circles). Pretreated or untreated effector cells were mixed with peptide 18I10-pulsed P815 cells at the indicated ratios.

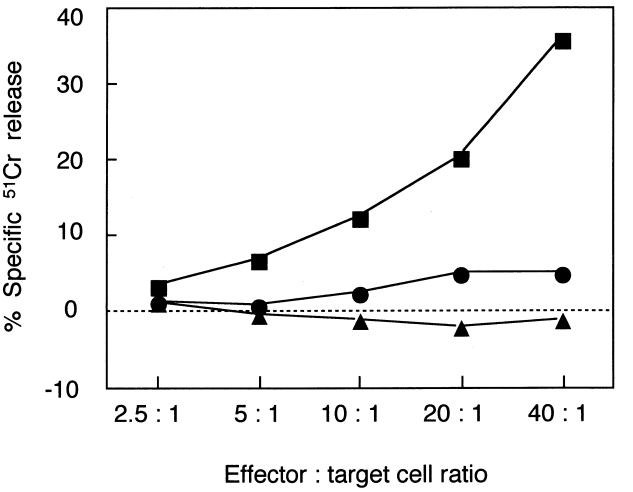

Fig. 3. The CTL response specific for the peptide is restricted to MHC class I. In order to examine MHC restriction of the CTL response induced in BALB/c (H-2^d) mice by oligomannose-coated liposomes containing peptide 18IIIB, P815 cells (H-2^d; squares), EL4 cells (T-lymphoma expressing H-2^b; circles), and BW5147 cells (T-lymphoma expressing H-2^k; triangles) were pulsed with peptide 18I10 and then used as target cells. Details of experimental procedures are described in Section 2.

It has been reported that mannan-coated liposomes containing a hybrid protein of gag and env of human T lymphotropic virus type 1 (HTLV-1) induce MHC class I-restricted and $CD8^+$ CTL specific for HTLV-1-positive cells in rats [21] and that DNA of HIV-1 incorporated into mannan-coated *N*-*t*-butyl-*N*′-tetradecyl-3-tetradecylaminopropionamidine or mannan-coated liposomes elicits HIV-specific CTL activity [22,23]. This finding (that mannan abundant in mannose residues is important in eliciting CTL response) is consistent with our finding that oligomannose residues in the neoglycolipids coated on liposomes are important. From the viewpoint of practical use, however, these are completely different. Mannan is highly immunogenic and toxic [24,25]. Mannan is known to be lethal when intravenously injected in mice [24] and to induce an antibody response and B-cell mitosis [25]. In addition, mannan-coated liposomes cause obvious microabscesses at the skin sites of subcutaneous inoculation [26]. In contrast, oligomannose-coated liposomes were not immunogenic or toxic.

The neoglycolipid used for coating of liposomes in the

present study is composed of innocuous lipid dipalmitoylphosphatidylethanolamine and mannopentaose. Its structure is included in the high mannose-type oligosaccharides of glycoproteins ubiquitously found in bodies of both mice and humans. Trials to generate antibodies against oligomannose by repeatedly injecting mice with the neoglycolipid containing the mannopentaose were unsuccessful. The mice remained healthy with no sign of adverse effects, suggesting that the neoglycolipid is not immunogenic or toxic. In the present study, it was also shown that liposomes that were oligomannose-coated with the neoglycolipid did not cause any damage to the skin at injection sites. From a practical point of view, therefore, use of liposomes that are oligomannose-coated with neoglycolipid offers a novel approach to designing a safe adjuvant for inducing effective CTL to control HIV infection and progression to AIDS, and development of other diseases.

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