Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2010, Article ID 242539, 8 pages
doi:10.1155/2010/242539

Review Article

Recent Advancements in Cytotoxic T Lymphocyte Generation Methods Using Carbohydrate-Coated Liposomes

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Received 29 January 2010; Accepted 22 April 2010

Academic Editor: Hanchun Yang

Both tumor-specific $CD4^+$ and $CD8^+$ T cells have been identified, and the latter is known as a major effector of adaptive antitumor immune responses. Optimal antitumor immune responses are considered to require the concomitant activation of both $CD8^+$ and $CD4^+$ T cells and the additional selective activation of $CD4^+$ T cells with helper, but not regulatory function. As optimal antitumor immune responses are generated by the concomitant activation of both T cell types, it is necessary for vaccine methods involving cytotoxic T-lymphocytes (CTLs) generation to possess a mechanism whereby antigen presenting cells can present administrated exogenous antigens on not only Major histocompatibility complex (MHC) class II, but also MHC class I molecules.

1. Introduction

We have previously reported the development of a new drug delivery system (DDS) based on the carbohydrate recognition properties of phagocytic cells to control metastatic cancer in extranodal lymphoid tissue of the abdominal cavity [1]. Further, we demonstrated that our DDS could be used for the induction of CTLs through the presentation of exogenous antigens on MHC class I molecules of phagocytic cells [2].

In accordance with findings from many attempts, including ours, to generate antigen-specific CTLs, this paper provides an overview of current trials of liposome-based vaccines. Furthermore, we discuss the feasibility concerning our vaccination technique by summarizing accumulated knowledge regarding receptor candidates.

2. Overview

In order to reject invading pathogens and cancer cells, expansion of T cells is known to be activated by small peptides on Major histocompatibility complex (MHC) class I or MHC class II molecules on the cell surface of antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages. We will mainly introduce recent progression of vaccine methods to generate $CD8^+$ cytotoxic T lymphocytes (CTLs) in this paper, while first mentioning the indispensable roles of $CD4^+$ helper T cells that support the expansion and persistence of CTLs [3–5]. Indeed, optimal antitumor immune responses are generated by the concomitant activation of both $CD8^+$ and $CD4^+$ T cells because of the selective activation of $CD4^+$ T cells with helper, but not regulatory functions [6]. Generally, exogenous antigens presented by MHC class II molecules are intended for $CD4^+$ T cells, whereas internal antigens from the cell itself, components of virus infected cells, and cancer antigens are presented on MHC class I molecules for activation and expansion of $CD8^+$ T cells. Consequently, it is necessary for vaccine development methods involving CTL generation to possess a mechanism whereby administrated exogenous antigens can be presented not only on MHC class II, but also class I molecules of APCs [6–8].

For vaccine development methods whereby exogenous antigens are exhibited both on MHC class I and class II molecules to induce antigen-specific $CD8^+$ and $CD4^+$ T cells,

our novel drug delivery system (DDS) using oligomannose-coated liposomes (OMLs) [1] that target phagocytic cells can be tailored for this purpose [2]. Indeed, a novel OML-based vaccine could reject transplanted tumor cells, prevent progression of encephalitis and vertical transmission, and reduce offspring mortality of *Neospora caninum* as shown in a feasibility study for its clinical use [2, 9–11].

OML-based vaccines produce strong adjuvanticity for CTLs. As liposomes coated by oligomannose are exclusively taken up by $F4/80^+$ intraperitoneal mononuclear cells and gathered at extranodal lymphoid tissues, the so-called "milky spots in abdominal cavity" [1], the underlining mechanisms of OML-based vaccine appear to be accompanied by an immune surveillance system for detecting pathogens invading the abdominal cavity in either a mannose dependant or mannose independent manner. Important roles for macrophages and complement systems are well known in the clearance of foreign materials, invading bacteria, and tumor cells from the abdominal cavity. Moreover, it is the milky spots that are the exact locus of this clearance process [12–15]. Taken together, a line of clearance process for OMLs may associate with strong adjuvanticity to induce CTLs.

Some diseases such as hepatitis C virus infection and malignancies still remain to have vaccine methods developed for them using disease-specific CTLs by elucidating their basic roles [5, 16, 17]. Many attempts to generate antigen-specific CTLs have been conducted, based on new experimental evidence. In accordance with these efforts, this paper will provide an overview of current trials concerning liposome-based vaccine delivery, and we discuss the feasibility of an OML-based vaccine based on recently accumulated knowledge of the carbohydrate recognition system as a target for OML-based vaccine delivery systems.

2.1. Liposome-Based Vaccine Delivery to Generate CTLs. Based on results from materials investigated for immunization, many types of liposomes have been tested for use in attempts to increase the effect of CTL generation against delivered vaccine antigens. New materials used in liposome preparation have been investigated to see whether they could effectively generate CTLs while monitoring the following three effects [18–20]: (1) an increase in the fusion efficiency between the cell membrane and liposomes, (2) the stabilization of liposomes in blood circulation, and (3) efficient delivery of vaccine antigens to APCs.

The approach tried first was to increase the fusion efficiency between the cell membrane and liposomes, because external antigens spilling from endosomes into the cytosol is considered to be the most important step for entry into the class I processing pathway for CTL generation [18, 21, 22]. To this purpose, use of peptide sequences referred to as antennapedia homeodomain [23, 24], and the hemolytic protein of *Listeria monocytogenes*, listeriolysin O [25], succeeded in enhancing the introduction of CTL epitopes into the class I processing pathway, resulting in the increased generation of CTLs. Furthermore, fusogenic liposomes prepared by fusing simple liposomes with Sendai virus particles can deliver encased antigens into the cytosol to generate CTLs [26].

Retaining liposomes in blood circulation is another way to increase the efficiency of CTL generation. Increasing retention time has been achieved by reducing surface-macromolecule interaction, which provides less opportunity for liposomes uptake by phagocytic cells and hepatocytes [27, 28]. The approach is highly effective for induction of CTLs against antigens encased in liposomes [29, 30]. To this purpose, polyethylene glycol (PEG)-modified lipids have become universally used in the preparation of liposomes (PEG-liposome) [29–31], which can more greatly enhance the generation of a $CD8^+$ T cell response than when given in soluble form or in conventional or positively charged liposomes [32]. Moreover, new lipids isolated from Archaea have also been used in the preparation of liposomes because of their stabilizing effect on liposomes in a manner similar to that for PEG-modified lipids. Archaea liposomes showed higher stability against extreme pH, oxidation, elevated temperatures, and action of lipases than conventional liposomes [33–35].

While higher stabilization in blood circulation increases CTL generation as discussed above, enhanced uptake by phagocytic cells has been indicated to elicit strong adjuvanticity to induce antigen-specific CTL responses [19]. The relevant examples of specific delivery of liposomes to phagocytic cells are cationic liposomes and OMLs. Positive charge on a liposome surface enhances uptake by APCs more than neutral membranes, and more robust immune responses for CTL generation and antibody production were seen in mice immunized using positively charged liposomes than with neutral liposomes [23, 28, 36–38]. To add positive charge to the surface of liposomes in earlier studies, cationic cholesterol derivatives such as 3β[N-(N′, N′-dimethylaminoethane)-carbamyl] cholesterol hydrochloride were frequently used.

Coating with ligands for pattern recognition receptors such as a mannose receptor (MR) on APCs is expected to have the analogous effect of adding positive charge to the liposome surface using cationic cholesterol derivatives, because ligand binding triggers endocytosis of liposomes by APCs such as DCs and macrophages [39, 40]. Related to this concept, Chikh et al. have indicated a line of phagocytic receptors for a variety of ligands, FCγ RI [41, 42], mannose [39, 40], $\alpha M\beta 2$ integrin (CD11b CD18) [43, 44], CD36, and $\alpha v\beta 5$ integrin [45], for forced uptake by APCs [19].

2.2. Carbohydrate Coatings on Liposomes to Deliver Vaccine Antigen to APCs. To induce robust immune responses using carbohydrates recognition by phagocytic cells, either mannose residues coupling on antigens or coating on an antigen encased liposomes appears to show promise. Using these methods, antigens were able to efficiently deliver phagocytic cells such as APCs, due to the uptake by mannose recognition receptors such as macrophage mannose receptor (MMR, CD206) and DC-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN) preferentially expressed on them, resulting in effective induction of CTLs [1, 46–50]. It may be associated with a nature of mannose residues, which significantly enhances immunogenicity of antigens and strongly promotes DC maturation through

TLR4 function [51]. Concerning carbohydrate coupling on antigen, not only the high-mannose oligosaccharide [47] and O-linked short mannose (2-3 mannoses) from *P. pastoris* [48–50], but also fucosylated oligosaccharides such as Lewis X or Lewis B [50], could be used to specifically deliver to dendritic cell by the other preferential binding specificity of DC-SIGN. Indeed, either mannosylated or fucosylated antigens could enhance CTLs responses depending on antigen presentation via class I molecules. [47, 49, 50].

Concerning mannose residues coating on liposome surfaces, we used mannotriose-dipalmitoylphosphatidylethanolamine (Man3-DPPE) [52] in the preparation of liposomes with dipalmitoyl phosphatidylcholine and cholesterol, at a molar ratio of 1 : 10 : 10 [52, 53]. Man3-DPPE, a neoglycolipid that is composed of Man3 and DPPE, is synthesized by reductive amination between an aldehyde group of oligosaccharides and the amino group of DPPE [52]. Because of the hydrophobic lipid moiety of the neoglycolipid, Man3-DPPE can easily be incorporated into the lipid bilayer of liposomes. The liposomes contain Man3-DPPE, and we have named them OMLs.

As mentioned in our previous studies, OMLs injected into the abdominal cavity are taken up by $CD11b^+$ phagocytic cells that deliver material to milky spots [1, 46]. Indeed, when 5-fluorouracil (5-FU) was encapsulated in the OMLs, more than 60% of the administered 5-FU accumulated in the omentum where milky spots gathered [1]. In other words, the OML-based DDS targets $CD11b^+$ phagocytic cells that act as cellular vehicles for material delivery. Recent use of hematopoietic or mesenchymal stem cells as cellular vehicles has led to significant progress in gene delivery techniques, while Burke has indicated the advantages of using phagocytic cells as natural cellular vehicles [54]. Phagocytic cells such as macrophages in the abdominal cavity take up large amounts of particles and accumulate them at not only lymphoid tissue, but also various pathological sites such as cancer lesions, wounds, atherosclerotic plaques, and arthritic joints [54]. Consequently, for delivery of materials, OMLs are a valuable tool for exploiting the nature of phagocytic cells.

We recently demonstrated the usefulness of OMLs as carriers for the delivery of vaccine antigen to generate and expand CTLs by employing ovalbumin (OVA) as a model cancer antigen [2]. Indeed, APC came to express OVA-derived peptides obtained by OML-based delivery in the context of both MHC class I and II molecules, which were evaluated by the detection of interferon gamma (IFNγ) production in the coculture with OML-delivered APCs and either $CD8^+$ or $CD4^+$ T cells from the spleens of T cell receptor transgenic mice OT-I (specific for H-2Kb/$OVA_{257–264}$) [55, 56] or OT-II (H-2Ab/$OVA_{323–339}$) [57], respectively [2]. Moreover, only the spleen cells from mice immunized with OML-OVA, but not bare liposomes without coating-encased OVA, showed cytotoxicity against E.G7-OVA, and only the mice preimmunized with subcutaneous challenge by OML-OVA rejected E.G7-OVA, but not EL4. These results together indicate that the OMLs can be used as an effective antigen delivery system for immunotherapy activating both CTL and Th subsets. OMLs are very useful not only for the promotion of nonglycosylated protein uptake by APCs, but also for the enhancement of antigen processing of encased antigens. This advantage of OML-mediated immunization will hopefully facilitate the simultaneous activation of tumor antigen-specific $CD4^+$ and $CD8^+$ T cells [2], and have the potential for use in cancer immune therapy [9].

It is well known that carbohydrates binding protein on APCs and complement lectin pathways recognize conserved motifs of glycans on pathogens. Carbohydrates binding proteins on phagocytic cells participate in the capture of materials to internalize, while the complement lectin pathway actively generates peptide fragments from C3, facilitating opsonophagocytosis by phagocytic cells through the complement receptors (CRs). Inhibition of complement component C3 and complement receptor type 3 (CR3, CD11b/CD18) could block the uptake process of OMLs by phagocytic cells [58, 59]. These observations support the hypothesis that carbohydrates binding receptors promote the uptake of liposomes in accordance with the activated lectin pathway, acting as an essential step in robust CTL responses against antigens encased in liposomes [59–61].

3. Possible Target Receptors on APCs Using Liposomes with Carbohydrate Coatings

In this section, we focus on receptor candidates on APCs for uptake of liposomes with carbohydrate coatings. Based on recent advancements in technologies to investigate structure–function relationships of glycans, knowledge about the properties of carbohydrate-binding proteins has dramatically increased, and offers their possible use as targets for the delivery of vaccine antigens. Here, we introduce DC-SIGN (CD209), MMR (CD206), and CRs to illustrate the possible mechanism for OML delivery to induce CTLs.

DC-SIGN (CD209) is a type II membrane protein, which is now established as a mannose-binding protein [62], and which appears to be a major receptor for OMLs [63]. It exhibits higher avidity to mannose through multimer formation, while there is not a one-to-one correspondence between the mannose and carbohydrate recognition domains (CRD) of DC-SIGN because of the binding specificity for *N*-acetylglucosamine (GlcNAc) and fructose (fuc) [62]. DC-SIGN was initially reported as having specific binding for HIV gp120 [64], and further, ICAM-2 and ICAM-3 on T cells were identified as ligands [65]. Because the interaction between DC-SIGN and ICAM-3 is inhibited by added free mannose, mannose residues of ICAM-3 act as ligands for CRD of DC-SIGN [65]. Though the ligand binding has been considered to enhance T cell activation by MHC class II (and possibly MHC class I) molecules in a restricted manner [66, 67], the exact role of how DC-SIGN associates to induce and activate CTLs remains to be elucidated.

The initial immunological role of MMR/CD206 has been considered to be for the surveillance of invading pathogens such as *Candida albicans* and *Pneumocystis carinii* [68, 69]. MMR has eight CRD/C-type lectin-like carbohydrate recognition domains, one fibronectin type II repeat domain (Fn-II D), five CRDs (CRD4–8), which bind with mannose, but also fuc and GlcNAc [70, 71]. Targeted delivery using a specific antibody to MMR increases the uptake of delivered

Table 1: Lectin-like receptors, complement receptors (CR), and ligands.

Receptors	Ligands
DC-SIGN (CD209)	Fucose, mannose, N-acetylglucosamine
DEC-205(CD205)	Unknown
Mannose receptor (CD206)	Fucose, mannose, N-acetylglucosamine
CR1 (CD35)	C3b, iC3b
CR2 (CD21)	C3d
CR3 (CD11b/CD18, Mac-1)	iC3b
CR4 (CD11 *c/CD18)*	iC3b
CR5 (CRlg, VSIG4)	iC3b

antigens by phagocytic cells, resulting in the concomitant activation of both $CD8^+$ and $CD4^+$ T cells through antigen presentation on MHC class I and class II molecules [39, 72]. This observation suggests a possible role of MMR in the induction of CTLs when OML is used for immunization.

Though DEC-205/CD205 has 10 CRDs and is highly homologous with MMR, its avidity to any glycans has not been detected. It may be caused by the limited number of glycan structures to perform binding analysis [73–75]. DEC-205 expresses exclusively on mature DCs, but for macrophage and immature DCs, targeting DEC-205 to deliver liposome-containing vaccine antigen has the potential to improve the efficiency of CTL generation because of possible adjuvanticity [76].

To date, five members of CRs (CR1-5) have been identified, and all of which are associated with opsonophagocytosis through the activation of the complement system [77]. Expression of all CRs has been exclusively detected on monocyte/macrophage lineage cells, while the distinct presence of CR1 and CR2 on erythrocytes and B cells is known [78]. Table 1 shows the differential binding specificity of each CR for C3 fragments [79–81].

CRs appear generally to have other functions besides facilitating opsonophagocytosis, known as their classical role. CRIg/VSIG4 is a recently identified fifth member (CR5) of the CR family, the long form of which is identical to Z39Ig reported in earlier studies [78, 81]. CR5, CRIg/VSIG4/Z39Ig, on monocytic cells can bind C3b and iC3b to internalize opsonized materials in the same way as other CRs, while it appears to play other roles, such as B7 family molecules, to suppress the activation and proliferation of CTLs [82, 83]. In addition, CR2/CD21 on B cells was demonstrated to transduce positive signals for antibody production upon complex formation with CD19 and CD81 [84]. Taking into account that CRs have another function besides internalization, CR3 (CD11b/CD18, Mac-1) [85] and CR4 (CD11b) may play some role in generating CTLs upon taking up OML [84, 85].

4. Possible Infectious Disease Targets for a Vaccine Strategy Using OMLs

In order to prevent manifestation of some diseases whose main effectors are CTLs, administration of recombinant antigen as a vaccine requires particular adjuvants to induce CTLs sufficiently to reject causative pathogens. To date, live vaccines are exclusively applied for universal prophylaxis of domestic animals, because they appear to have higher efficacy for illness given infection than either recombinant or inactivated vaccine containing adjuvant because of the advantage of induction efficiency of CTLs. Because vaccination with OMLs is expected to induce concomitant activation of both CTLs and Th1 cells, it has the potential to alter general approaches for infection control in domestic animals from live vaccines to recombinant antigen.

Based on increasing knowledge regarding OML-based vaccines, we have attempted to use them for control of some infectious diseases. Promising results using OMLs could be obtained for infection by *N. caninum* [10] and *Leishmania major* [53] using animal models. By administration of *Neospora* antigen NcGRA7 encased in OMLs to mice, NcGRA7-specific Th1 cells were generated, preventing the transition of the infection to the brain and transplacental vertical transmission [10]. Moreover, administration of apical membrane antigen 1 of *Neospora* using OMLs succeeded to reduce offspring mortality [11]. Whether the induced CTLs eliminated *N. caninum* in infected mice, it remained to determine increased numbers of the infection-specific CTLs by ELISpot assay, and estimate disease activity using genetically engineered protozoans expressing OVA as surrogate antigens. For the *L. major* infection, as earlier models with genetically engineered protozoans showed important roles of CTLs in the elimination of *L. major* [86] similar to the intracellular protozoan parasite *Toxoplasma gondii* [87], both antigen specific CTLs and Th1 cells induced by OML vaccination would show efficacy in preventing development of the illness.

We consider that CTL generation by OML-based vaccines can also be applied to *Theileriosis*. *Theileriosis* is a serious infection in cattle caused by tick-borne parasites, and is classified as a lymphoproliferative or hemoproliferative disease, depending on the principal pathogenic feature. *T. parva* and *T. annulata* are agents of lymphoproliferative *Theileriosis* [88, 89], and CTLs against *T. annulata* and *T. parva* are known to prevent disease progression [90, 91]. To date, live vaccine is known to be efficacious as a prophylactic agent. Administration of either *T. annulata* attenuated by long time in vitro culture or the infection-and-treatment method for *T. parva* [92, 93] are known as effective vaccination methods preventing the development of the lymphoproliferative disease. These results suggest that

it is possible to use OML vaccines to prevent the onset of lymphoproliferative *Theileriosis*.

5. Conclusion

In accordance with the findings from many attempts to generate antigen-specific CTLs, this paper presents an overview of current trials concerning liposome-based vaccine delivery. We discuss the feasibility of using OML-based vaccine based on recently accumulated knowledge regarding carbohydrate recognition systems as targets for OML-based vaccine delivery systems. In order to practically use OML-based vaccine to introduce CTLs for prophylaxis of some infectious diseases, general use of ELISpot assays may be needed to monitor the efficacy of CTL generation. Taking into account the recent trend of surveying for latent infection of *Mycobacterium tuberculosis* [94] using this method, universal use of OML-based vaccine to induce CTLs is not limited by this issue. Further studies characterizing the type of immune response induced by OML-based vaccine delivery in cattle are planned and should provide additional insights for the optimal development of OML-based vaccine to generate CTLs.

Acknowledgments

Introducing our works was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and the New Energy and Industrial Technology Development Organization (NEDO) of Japan. The cost of publication of this paper was defrayed in part by payment of page charges. This paper must therefore be marked as an advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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