In this paper, we employed 3D structures constructed by a homology modeling method to map amino acid residues on the antigenic sites of HA. When compared to the presentation of simple primary sequences, the 3D presentation has following advantages: (a) There are several amino acid residues that are buried beneath the surface of the HA molecule, even if they are included in the antigenic sites described by the primary amino acid sequences. Since such amino acid residues do not directly contribute to the interaction with antibodies, the surface structures of antigenic sites that are accessible for antibodies can be compared more precisely in the presentation by 3D models than by the primary amino acid sequence. (b) An epitope likely consists of multiple amino acid residues belonging to different antigenic regions presented by the primary amino acid sequence. Such conformational epitopes can be illustrated only by the 3D presentation. (c) One of the purposes of this study is to provide a structural basis to confirm antigenic similarity between the 1918 H1N1 and the pandemic 2009 H1N1 viruses. For this purpose, we employed a homology modeling method rather than simply mapping on the existing crystal structure (e.g. 1918 H1N1 HA), since this method is generally used to generate a 3D structure of a protein molecule if there is no available crystal structure of the target protein [14]. Thus, we believe that this method produces more likely HA structure models of the viruses whose HA crystal structure are not available (e.g. CA2009). In fact, our homology modeling approach suggests that several amino acid residues were occasionally buried beneath or exposed to the surface of HA molecule, depending on the substitutions found in the viruses examined (Figure 1B and Figure S1). The homology modeling approach might enable us to analyze such dynamics of antigenic changes at molecular levels.

Our analysis indicated that 2009 H1N1 had undergone less significant antigenic changes of HA in the pig population than human H1N1 virus since their emergence in the early 1900s. The Centers for Disease Control and Prevention reported that vaccination with recent (2005–2009) human H1N1 viruses was unlikely to provide protection against 2009 H1N1 [15]; however, cross-reactive antibodies were detected in 33% of people aged 60 and over. Another report showed that appreciable neutralizing antibodies against CA2009 were present in the sera collected from individuals born before 1918 [16]. Our 3D models provide a protein-structural basis supporting these observations, and further suggest that infection with the 1918 H1N1 or early human H1N1 viruses (viruses present before the 1940s), but not with antigenically divergent human H1N1 viruses circulating after the 1950s, elicited cross-neutralizing antibodies to 2009 H1N1.

This virus will soon be subjected to complex immunological selection pressure by the antibody response that will be induced in the human population by vaccination and/or natural infection with homologous viruses, and pre-existing immunity cross-reactive to the early descendants of 1918 H1N1. In the present study, we showed that the antigenic structure of 2009 H1N1 HA might still be similar, at least in part, to that of the 1918 H1N1 HA. We speculate that the 2009 H1N1 HA antigenic sites involving the conserved amino acids will soon be targeted by neutralizing antibodies in humans. Thus, it is of interest to monitor whether these antigenic sites of 2009 H1N1 will undergo similar patterns of amino acid substitutions to those seen in seasonal H1N1 viruses during its epidemic period (Figure 4). Interestingly, we found that some of the recent variants of the 2009 H1N1 virus (as of November 3, 2009) have indeed undergone substitutions identical to those predicted in Figure 4. Although the present study still needs to be supported by experimental data, our approach may provide new perspectives on collective immunity against 2009 H1N1 and an insight into future antigenic changes of this new human pandemic influenza virus.

Methods

Sequence Data of HA Genes

Nucleotide sequences for HA genes of SC1918 (AF117241), BR2007 (CY030230), CA2009 (FJ966082), A/Puerto Rico/8/1934/Mount Sinai (AF389118), A/Bellamy/1942 (CY009276), A/Albany/4836/1950 (CY021701), A/USSR/90/1977 (DQ508897), A/Singapore/6/1986 (CY020477), A/Texas/36/1991 (AY289927), and A/Hong Kong/1035/1998 (AF386777) [2,3,17,18,19,20] were obtained from Influenza Virus Resource at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html).

Molecular Modeling

MODELLER 9v6 [21] was used for homology modeling of HA structures. After one hundred models of the HA trimer were generated, the model was chosen by a combination of the MODELLER objective function value and the discrete optimized protein energy (DOPE) statistical potential score [22]. After addition of hydrogen atoms, the model was refined by energy minimization (EM) with the minimization protocols in the Discovery Studio 2.1 software package (Accelrys, San Diego, CA) using a CHARMm force field. Steepest descent followed by conjugate gradient minimizations was carried out until the root mean square (rms) gradient was less than or equal to 0.01 kcal/mol/Å. The generalized Born implicit solvent model [23,24] was used to model the effects of solvation. The HA model was finally evaluated by using PROCHECK [25], WHATCHECK [26], and VERIFY-3D [27]. All figures are shown as a solvent-accessible surface representation prepared by PyMOL (DeLano Scientific LLC) [28]. All HA structures constructed by a homology modeling method are available in Supplementary Files S1, S2, S3, S4, S5, S6, S7, and S8.

Sequence Data Analyses for *N*-Glycosylation Sites

Custom-made programs were developed with the Ruby language and used for investigating the numbers of potential *N*-glycosylation sites and candidate codons (*Cand1*) in HA sequences. The programs are available upon request.

Supporting Information

Table S1
Found at: doi:10.1371/journal.pone.0008553.s001 (0.04 MB PDF)

Figure S1 Amino acid substitutions of seasonal human H1N1 virus HAs shown in close-up views of each antigenic site. The strains used in this analysis are corresponding to those shown in Figure 2. Amino acids are colored according to the scheme in the legend of Figure 1B.
Found at: doi:10.1371/journal.pone.0008553.s002 (1.02 MB PDF)

File S1 PDB file of the homology model of H1 HA (A/California/04/2009) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s003 (0.20 MB ZIP)

File S2 PDB file of the homology model of H1 HA (A/Bellamy/1942) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s004 (0.20 MB ZIP)

File S3 PDB file of the homology model of H1 HA (A/Albany/4836/1950) after energy minimizations.

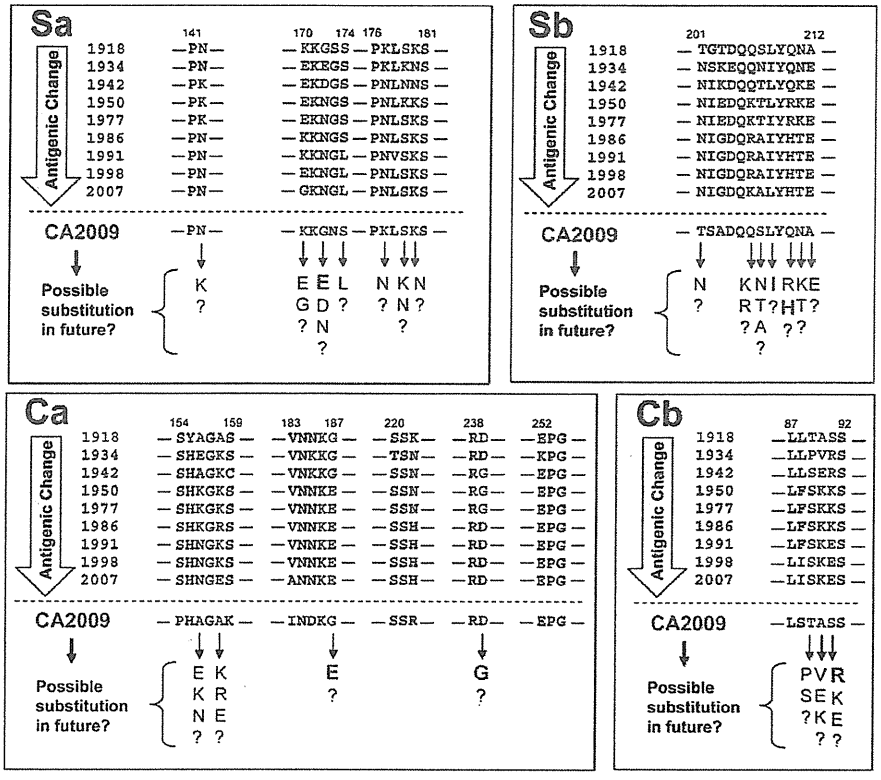

Figure 4. Prediction of the future amino acid substitutions on the antigenic sites of 2009 H1N1 HA. Amino acid sequences of HA antigenic sites of human H1N1 viruses are shown. Sequence data are corresponding to those of virus strains shown in Figures 1 and 2. Amino acid residues shared between 1918 H1N1 (SC1918) and 2009 H1N1 (CA2009) are shown in red, and those that have been substituted since 1934 are shown in blue. Amino acid residues indicated by arrows represent the predicted substitutions which might be associated with antigenic changes of 2009 H1N1 in the near future. The amino acid substitutions which have already been found in the recent variants of the 2009 H1N1 virus (as of November 3, 2009) are shown in bold pink letters.
doi:10.1371/journal.pone.0008553.g004

Found at: doi:10.1371/journal.pone.0008553.s005 (0.20 MB ZIP)

File S4 PDB file of the homology model of H1 HA (A/USSR/90/1977) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s006 (0.20 MB ZIP)

File S5 PDB file of the homology model of H1 HA (A/Singapore/6/1986) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s007 (0.20 MB ZIP)

File S6 PDB file of the homology model of H1 HA (A/Texas/36/1991) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s008 (0.20 MB ZIP)

File S7 PDB file of the homology model of H1 HA (A/Hong Kong/1035/1998) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s009 (0.20 MB ZIP)

File S8 PDB file of the homology model of H1 HA (A/Brisbane/59/2007) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s010 (0.20 MB ZIP)

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Author Contributions

Conceived and designed the experiments: MI KI HK AT. Analyzed the data: MI RY DT. Wrote the paper: MI AT.

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今日の新型インフルエンザワクチンの問題点と将来展望

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キーワード●インフルエンザワクチン，液性免疫，細胞性免疫，リポソーム

現行のインフルエンザワクチンの問題点

2009年にブタを起源としてヒトからヒトへ感染する能力をもった新型インフルエンザ(A/H1N1)ウイルス[swine-origin influenza A (H1N1) virus；S-OIV]が出現し，地球規模で感染が拡大した．この背景には，現行の季節性インフルエンザに対するワクチンが新型インフルエンザ(A/H1N1)ウイルスの感染予防には奏効しないという問題がある．

現行のインフルエンザワクチンはインフルエンザウイルス表面のHA(hemagglutinin；ヘマグルチニン)抗原に対する抗体の産生を誘導し，ウイルスが宿主の細胞に吸着するのを阻止することを目的としている．一方で，インフルエンザウイルスには表面抗原の異なる複数の亜型が存在し，HA(16種類)，NA(neuraminidase；ノイラミニダーゼ．9種類)の組み合わせにより，理論的には144通りのウイルス亜型が想定される．

抗原と抗体とはいわゆる「鍵と鍵穴」の関係にあるため，1つのウイルス亜型に結合する抗体は他のウイルス亜型には結合しない．また，同一のウイルス亜型においても，HAの蛋白構造の一部が遺伝子変異を起こすことにより抗体が結合できない場合もある．新型インフルエンザ(A/H1N1)ウイルスが季節性インフルエンザウイルスと同じH1N1亜型であるにもかかわらず，現行の季節性インフルエンザワクチンが奏効しないのはこのためである．

インフルエンザウイルスは2通りの方法で変異する．1つは，ウイルスに感染した宿主の体内でHA，NAの組み替え(遺伝子再集合)が行われて新しい亜型が出現する，いわゆる"antigenic shift"と呼ばれるもので，もう1つは，同一の亜型のなかで遺伝子変異体が生じる，いわゆる"antigenic drift"と呼ばれるものである．2009年の新型インフルエンザ(A/H1N1)ウイルスのHAには，季節性のH1N1インフルエンザウイルスのHAと比較して約20～24%の遺伝子変異部分があることが知られている[1]．

米国CDC(Centers for Disease Control and Prevention；疾病予防管理センター)による調査の結果，季節性インフルエンザワクチンによって誘導される抗体は，新型インフルエンザ(A/H1N1)ウイルスに作用しないことが確かめられている[2]．これは，同一のHA亜型(H1)における遺伝子変異が原因であり，季節性インフルエンザウイルスのHAに対する抗体が新型インフルエンザ(A/H1N1)ウイルスのHAには結合しにくいことによると考えられる．

The Point at Issue of Currently Approved Influenza Vaccine and Future Aspect
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表1　異なるインフルエンザウイルス亜型におけるアミノ酸の相同性

分節	アミノ酸残基数	H5N1 (A/HongKong/156/97)	H1N1 (A/IvPR8/34)	H3N2 (A/Aichi/2/68)
M1	252	100%	95%	96%
M2	97	100%	86%	89%
NP	498	100%	94%	95%
NS1	225	100%	92%	88%
PA	716	100%	96%	96%
PB1	757	100%	97%	96%
PB2	759	100%	96%	96%

H5N1型（A/HongKong/156/97）株におけるウイルス内部蛋白のアミノ酸組成に対するH1N1型（A/IvPR8/34），H3N2型（A/Aichi/2/68）ウイルスのアミノ酸相同性（%）を示す．

このように，現行のインフルエンザワクチンは異なるウイルス亜型に対してだけでなく，同一の亜型内でもHAに遺伝子変異が起きたものに対しては十分に奏効しない場合が少なくないため，新型インフルエンザウイルスが出現するたびに新しくワクチンを製造する必要が生じる．

II「液性免疫」と「細胞性免疫」

上述のように，現行のインフルエンザワクチンはウイルスの表面に存在する抗原に対する抗体の産生（液性免疫）を誘導するが，生体にはこのほかに，ウイルスに感染した細胞を攻撃・除去することによりウイルスの複製を阻止する機能（細胞性免疫）も備わっており，ウイルスが自然感染した際に生体は液性免疫と細胞性免疫の双方を用いて感染防御を行っていると考えられている．液性免疫がウイルスの表面抗原を標的とするのに対して，細胞性免疫はウイルスの内部構造を含む全蛋白抗原を標的とすることができる．

インフルエンザウイルスにおいてはウイルス表面の蛋白抗原が頻繁に変異するのに対し，ウイルス内部を構成する蛋白は変異が少なく安定しており，ウイルス亜型間で相同性が高いことが知られている（表1）．このことから，インフルエンザウイルス内部の蛋白に特異的な細胞性免疫を誘導することにより，異なるウイルス亜型に対して有効性を発揮することのできるワクチンを創製することが期待されている[3]．

III 細胞性免疫誘導型インフルエンザワクチンの創製

一般に，ワクチンとして生体に投与された「外来抗原」は，抗原提供細胞によって主要組織適合複合体（MHC；major histocompatibility complex）クラスIIを介してCD4陽性T細胞に呈示された結果，液性免疫（抗体産生）が誘導される．従来のワクチンが主として抗体産生を誘導するのはこのことによる．一方，ウイルス感染細胞におけるウイルス由来抗原のような「内在性抗原」は，MHCクラスIを介してCD8陽性T細胞に呈示された結果，細胞性免疫（CTL；cytotoxic T lymphocytes，細胞傷害性T細胞）が誘導される．

また，外来抗原であっても抗原提供細胞において内在性抗原のように扱われ，MHCクラスIを介してCD8陽性T細胞に抗原呈示される，いわゆる「クロスプレゼンテーション」と呼ばれる経路が存在することが知られている．この経路を用いてワクチン抗原をCD8陽性T細胞に呈示させることができれば，CTLを誘導する

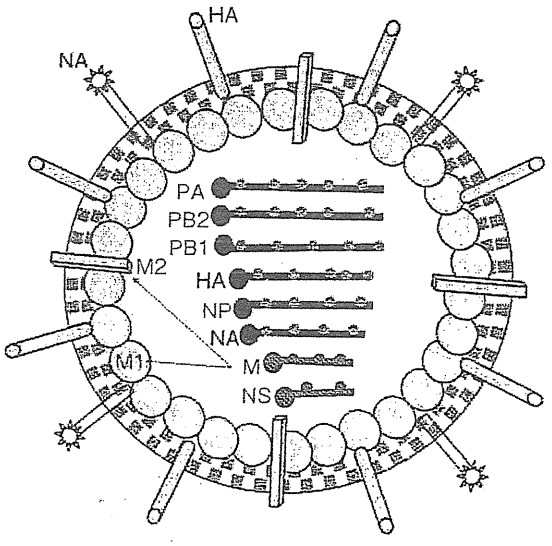

図1　インフルエンザウイルスの構造

ワクチンの創製が可能となる.

われわれはこれまでに，抗原をリポソームの表面に化学結合させてマウスに免疫することにより，高効率に抗原特異的IgG抗体産生が誘導される一方で，ワクチンに対するアレルギー反応の原因となるIgE抗体の産生が全く誘導されないことを見出した[4]．さらに，使用するリポソームの脂質組成を選択することにより，リポソーム表面に結合した抗原が抗原提供細胞によってCD8陽性T細胞に呈示され，抗原に特異的なCTLが誘導されることを見出した[5]．そこで，この抗原-リポソーム結合物を用いてCTL誘導型インフルエンザワクチンを創製することを試みた.

インフルエンザウイルスのゲノムを構成する8分節のうち，ウイルス表面に存在するHA，NA以外の6分節からCTLの標的となりうるアミノ酸配列（ペプチド）を選び出し，このペプチドを人工合成してリポソームに結合させた（図1）．この結合物をヒトMHCクラスIを発現したトランスジェニックマウス（HHDマウス）に投与したところ，高効率にCTLを誘導するペプチドが複数見付かり，その多くがH1N1型，H3N2型インフルエンザウイルスに共通に含まれるものであった.

さらに，このペプチド-リポソーム結合物で免疫したHHDマウスにインフルエンザウイルスを感染させたところ，感染後の肺におけるウイルスの増殖が非免疫対照群と比較して顕著に抑制されることがH1N1型およびH3N2型ウイルスにおいて確かめられた．この検討に用いたH1N1型ウイルス（PR8）はマウスに対して致死性を有するため，非免疫対照群では，すべてのマウスがウイルス感染後10日以内に顕著な体重減少の後に死亡したが，ペプチド-リポソーム結合物投与群では，すべてのマウスが体重減少もみられず無徴候で生存した[6]．

以上の結果から，インフルエンザウイルスの内部蛋白由来ペプチドを用いたCTL誘導型リポソームワクチンが，異なるインフルエンザウイルス亜型に対して有効であることが示された．この検討に用いたインフルエンザウイルス由来ペプチドは2009年の新型インフルエンザ（A/H1N1）ウイルスにも含まれるものであることから，単一のワクチンで季節性インフルエンザウイルスに対してだけでなく，新型インフルエンザ（A/H1N1）ウイルスに対する感染抵抗性をも誘導可能であることが予想される.

これまでの検討は，HLA-A2およびA24に拘束性のペプチドを用いて行われた．これらのHLA遺伝子型は日本人および欧米人の約8割に発現している．全人口の8割にインフルエンザウイルス感染抵抗性を誘導することができれば公衆衛生学的には有意義であるといえるが，われわれは現在，その他のHLA遺伝子型にも対応し，グローバルに有効なワクチンの開発を試みている.

おわりに

本稿で紹介したCTL誘導型インフルエンザワクチンは，従来のインフルエンザワクチンに取って代わろうというものではない．上述のように，生体には「液性免疫」と「細胞性免疫」と

いう２通りの免疫機能が備わっているが，従来のワクチンは主として液性免疫（抗体産生）を利用するものであった．

しかし，ウイルスのなかには変異することによって抗体による免疫応答から逃れるものがあり，そういった場合にはウイルス内部の変異しにくい部分を標的とした細胞性免疫（CTL）を誘導するワクチンが補完的に働くことが期待される．その代表例がインフルエンザであり，他にエイズ，C型肝炎，エボラ出血熱等の疾患を予防するワクチンの創製への応用が考えられる．

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REVIEW

Development of a cytotoxic T-lymphocyte-based, broadly protective influenza vaccine

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ABSTRACT

The current vaccination strategy against influenza is to induce production of antibodies directed against the surface antigens of these viruses. However, frequent changes in the surface antigens of influenza viruses allow them to avoid antibody-mediated immunity. On the other hand, it is known that cytotoxic T-lymphocyte (CTL) populations directed against internal antigens of influenza A virus are broadly cross-reactive to influenza virus subtypes. The present authors have previously demonstrated that antigens chemically coupled to the surface of liposomes made using unsaturated fatty acids are cross-presented by APCs via MHC class I to $CD8^+$ T cells and induce antigen-specific CTLs. Based on this finding, a liposome vaccine that is capable of inducing CTL response against internal antigens of influenza viruses and removing virus-infected cells in the host has been developed. The CTL-based liposomal technique might be applicable for developing vaccines against influenza and other viruses, such as hepatitis C, HIV, and severe acute respiratory syndrome corona virus, which frequently change their surface antigenic molecules.

Key words cytoxic T lymphocyte, influenza, liposome, vaccine.

Adjuvants are indispensable for viral vaccines, such as inactivated, subunit, or component vaccines, in which the antigens possess weak immunogenicity. However, the currently used aluminum adjuvants are known to stimulate only humoral responses (1) and are also known to induce IgE antibody production, which elicits an allergic response in some individuals following vaccination (2). Therefore, there is a need for improved adjuvants that are suitable for clinical use. Because they are known to act as powerful adjuvants when physically associated with a protein antigen, liposomes as antigen carriers (vehicles) have been particularly attracting attention among the candidates for adjuvants for novel vaccines (3–6). Most of the liposomal vaccines which have been proposed have been prepared by antigen entrapment within the aqueous lumen of liposomes (7). However, it is known that encapsulated and surface-linked liposomal antigens induce differential immune responses in humoral- (8) and cellular (9) immunity.

We have previously reported that surface-linked liposomal antigens induce IgE-selective unresponsiveness (10). Our results were consistent even when different procedures for coupling antigens with liposomes (11), or for producing liposomes with different lipid components (12), were employed. During the course of an investigation intended to clarify the mechanism of IgE-selective unresponsiveness induced by surface-coupled liposomal antigens, we discovered an alternative approach to regulating the production of IgE, one that is independent of the activity of T cells (13). The IgE-selective unresponsiveness

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List of Abbreviations: APCs, antigen presenting cells; CD, cluster of differentiation; CpG, cytosine-phosphate-guanine; CTL, cytotoxic T lymphocyte; DC, dendritic cell; IFN, interferon; HA, hemagglutinin; HLA, human leukocyte antigen; ICS, intracellular cytokine staining; IL, interleukin; MHC, major histocompatibility complex; NP, nucleoprotein; OVA, ovalbumin; S-OIV, swine-origin influenza (H1N1) virus; TLR, Toll-like receptor.

induced by the liposomal antigen involved direct effects on IgE, but not IgG switching *in vivo*. Thus, we expected that surface-linked liposomal antigen would be suitable for the development of a novel vaccine which would induce minimal IgE synthesis. Moreover, given the relatively low allergic response to, and increased antigenicity of, this allergen, this form of antigen preparation would be suitable for allergen immunotherapy (14, 15). In addition, we recently found that when lipid components are chosen for liposomes, surface-coupled liposomal antigens are cross-presented to $CD8^+$ T cells via MHC class I (16). Therefore, surface-linked liposomal antigens might be suitable for the development of tumor vaccines that would present tumor antigens to APCs and induce antitumor responses, and for the development of virus vaccines that would induce CTLs to eliminate virus-infected host cells.

A novel S-OIV which was first detected in Mexico in March 2009 spread globally via human-to-human contact. Although S-OIV belongs to the same HA serotype as seasonal human H1N1 influenza virus, vaccination with seasonal influenza vaccine does not result in cross-reactive antibodies (17), possibly because there is a wide protein sequence divergence of around 20–24% between S-OIV H1 and human seasonal influenza H1 (18). The current vaccination strategy is to elicit neutralizing antibody responses against HA, a surface glycoprotein of influenza viruses. Although antibody-mediated immunity to the surfaces of viruses reduces the probability of infection and morbidity, an antibody against one influenza virus type or subtype provides limited or no protection against another. Moreover, as a consequence of the frequent development of antigenic variants through antigenic drift, an antibody to one antigenic variant of an influenza virus might not protect against a new antigenic variant of the same type or subtype. Therefore, although vaccines designed to induce antibodies against HA provide reasonable protection against homologous viruses, it is feared that, due to the high rate of viral diversification, the vaccines currently being produced may have HA sequences so different from those of any pandemic strain that they would have little or no efficacy (19).

In natural infection, it is known that the host responds by inducing humoral and cellular immunity against the pathogen. Humoral immune responses consist of production of antibodies that bind to the surfaces of bacteria and viruses, whereas cellular immune responses mediate immunity to intracellular pathogens. Since Effros *et al.* reported in 1977 that influenza A virus-specific CTLs are broadly cross-reactive for cells of the same MHC class I type infected with serologically distinct H1N1 and H3N2 influenza viruses (20), it has been known that CTLs specific to internal proteins show high cross-reactivity between strains and between subtypes, reflecting more than 90% conservation of the internal proteins (21). Lee *et al.* recently reported that memory T-cells established by seasonal human influenza A infection cross-react with H5N1 in healthy individuals who have not been exposed to H5N1 viruses (22). The $CD8^+$ T-cells of the participants recognized multiple synthesized influenza peptides, including peptides from the H5N1 strain. Thus, vaccine formulas that include heterosubtypic T-cell-mediated immunity might confer broad protection against avian and human influenza A viruses.

In general, extracellular antigens are presented via MHC class II molecules to $CD4^+$ T-cells, whereas intracellular antigens are presented via MHC class I molecules to $CD8^+$ T-cells. To induce influenza-specific CTLs, vaccine antigens must be loaded onto the class I MHC processing pathway in the APCs via cross-presentation. In the process of cross-presentation, exogenous proteins cross over to the endogenous pathway, thus gaining access to MHC class I molecules (23). However, the currently approved alum adjuvant, which was first described by Glenny *et al.* in 1926 (24), is known to be effective only for induction of humoral immunity, and not for induction of cell-mediated immunity (25). Consequently, development of a novel vaccine adjuvant is essential for production of a $CD8^+$ T-cell vaccine.

Since liposomal conjugates induce CTLs efficiently when CTL epitope peptides are coupled to the surfaces of liposomes (16), they were expected to be suitable for development of peptide vaccines (26) . We recently demonstrated that liposome-coupled peptide $NP_{366–374}$, derived from NP of H3N2 influenza virus induces antigen-specific CTLs and successfully suppresses replication of H3N2 influenza virus in the lung in C57BL/6 mice (27). We further evaluated a possible application of liposome-coupled peptides to the development of an influenza vaccine using HLA-A*0201-restricted CTL epitope peptides and HLA-A*0201-transgenic mice.

In this review, we summarize data indicating that a correlation exists between the lipid component of liposomes and the immune response induced by surface-linked liposomal antigens, and discuss the potential of surface-linked liposomal antigens for the development of CTL-based viral vaccines.

Liposomes with different lipid components exert different adjuvanticity in antigen-liposome conjugates via differential recognition by antigen presenting cells

Based on our previous finding that liposomes with different lipid components display different adjuvant effects when antigens are coupled with liposomes via glutaraldehyde (12), we investigated adding antigen-liposome

conjugates prepared using liposomes with different lipid components to a macrophage culture, and phagocytosis and digestion of liposome-coupled antigen by the macrophages (28). When mice were immunized with OVA-liposome conjugates containing liposomes consisting of either saturated- or unsaturated fatty acids, the degree of anti-OVA IgG antibody production induced by these two OVA-liposome conjugates was significantly different; OVA-liposome conjugates made using the "unsaturated" liposomes induced more than ten-fold greater anti-OVA IgG production than that induced by OVA-liposome conjugates made using "saturated" liposomes, whereas the same dose of plain OVA solution induced far less anti-OVA IgG antibody production than that induced by OVA-liposome conjugates.

In order to examine whether the different adjuvant effects of these two liposome preparations were due to differential recognition of liposomal antigens by APCs, we investigated phagocytosis of OVA-liposome conjugates by macrophages by adding fluorescence-labeled OVA coupled with "saturated" or "unsaturated" liposomes to the macrophage culture. We found that more OVA were incorporated by macrophages when OVA were coupled to "unsaturated" liposomes than when OVA were coupled to "saturated" liposomes. To compare the processing of OVA coupled either with "saturated" or "unsaturated" liposomes by macrophages, we further investigated the fluorescence intensity of the macrophages by adding DQ-OVA-coupled liposomes to the macrophage culture. DQ-OVA exhibits green fluorescence upon proteolytic degradation. It appeared that the amount of OVA processed by macrophages in the same time period was greater when OVA was coupled to the "unsaturated" liposomes than when it was coupled to the "saturated" liposomes.

We further investigated antigen presentation by macrophages to an antigen-specific T-cell clone using the same set of conjugates. When we cultured macrophages in the presence of OVA-liposome conjugates prior to co-culture with the OVA-specific T-cell clone, 42–6A, significantly greater IL-2 production was observed when the macrophages were pre-cultured with OVA-liposomes made using "unsaturated" liposomes. Although a substantial amount of IL-2 was produced when the macrophages were pre-cultured with OVA-liposome conjugates made using "saturated" liposomes, the amount of IL-2 was still more than ten-fold less than that in the "unsaturated" liposome group.

Thus, these results clearly demonstrate that the adjuvant activity of liposomes observed primarily *in vivo* is closely correlated with recognition of antigen-liposome conjugates and the presentation of liposome-coupled antigen by macrophages, suggesting that the adjuvant effects of liposomes are exerted at the beginning of the immune response, that is, upon recognition of the antigen by APCs.

Antigens coupled to the surface of liposomes made using unsaturated fatty acid are cross-presented to $CD8^+$ T cells and induce potent antitumor immunity

Liposomes with different lipid components have been demonstrated to display different adjuvant effects when antigens are chemically coupled to their surfaces (12, 28). Here, we investigated the antigen presentation of liposome-coupled OVA *in vitro*, and found that OVA coupled to "unsaturated" liposomes are presented to both $CD4^+$ and $CD8^+$ T-cells while OVA coupled to "saturated" liposomes are presented only to $CD4^+$ T cells (16). When we co-cultured APCs derived from BALB/c mice with OVA-liposome conjugates made using liposomes with the above two different lipid components, and subsequently cultured them with splenic $CD4^+$ or $CD8^+$ T cells of OVA-immune BALB/c mice, OVA-liposome conjugates with the two types of liposome induced production of comparable amounts of IFN-γ by $CD4^+$ T cells. However, OVA-liposome conjugates made using "saturated" liposomes did not induce IFN-γ production by $CD8^+$ T cells, while OVA-liposome conjugates made using "unsaturated" liposomes induced significant production of IFN-γ.

In most APCs, exogenous antigens cannot be presented via the MHC class I pathway because exogenous antigens are unable to gain access to the cytosolic compartment. This segregation of exogenous antigens from the class I pathway is important in preventing CTLs from killing healthy cells that have been exposed to foreign antigens but have not become infected (29). Consequently, in general, exogenous antigens do not prime CTL responses *in vivo*. However, there are several exceptions to this rule, reflecting an ability of the antigens to be delivered into the cytosolic compartments in some cases (30–34). We have shown that antigens coupled to "unsaturated" liposomes are presented to both $CD4^+$ and $CD8^+$ T cells. Confocal laser scanning microscopic analysis demonstrated that a portion of the OVA coupled to liposomes received processing beyond the MHC class II compartment, suggesting that degradation of OVA occurs in the cytosol, and that peptides generated in this manner are presented to $CD8^+$ T cells via MHC class I. We further confirmed cross-presentation induced by OVA coupled to liposomes consisting of unsaturated fatty acids in *in vivo* CTL induction experiments. CTLs were successfully induced *in vivo* only when OVA or $OVA_{257–264}$ chemically coupled to "unsaturated" liposomes was inoculated into mice.

We further investigated the ability of antigen-liposome conjugates to induce antitumor immunity. The aim of cancer vaccination is to generate an immune-mediated anti-tumor associated antigen response that results in elimination of the tumor. The antigen of choice may be the whole protein alone or one with immune stimulatory components, or defined epitopes (e.g., peptides), of the target antigen (35). Recent preclinical studies have demonstrated that combined therapies involving the use of vaccines with cytokines, activators of DCs such as TLR ligands or mAb to CD40, or recombinant vectors that provide a stimulus to the innate immune system, result in enhanced antitumor responses (36). In this study, antigenic peptides were chemically coupled to the surface of liposomes and inoculated into tumor-bearing mice in combination with CpG and anti-IL-10 monoclonal antibodies. This treatment successfully induced eradication of the tumor mass, whereas inoculation of mice with CpG and anti-IL-10 monoclonal antibodies with peptide solution containing the same amount of $OVA_{257-264}$ as liposome-coupled $OVA_{257-264}$ did not affect E.G7 tumor growth. It has been reported that CpG and the anti-IL-10 receptor antibody reverse tumor-induced DC paralysis, resulting in tumor rejection by CTL activated by DC (37). These results suggest that, by choosing appropriate lipid components for liposomes, tumor vaccines that use surface-coupled liposomal antigens to present tumor antigens to APCs and induce antitumor responses might be developed.

Thus, in contrast to previous investigations of liposome-based drug-delivery systems which have focused on encapsulation of antigens into liposomes, the potential of surface-linked liposomal antigens for application to vaccine development has been investigated (7, 38). During the course of this investigation, several advantages of liposome-coupled antigens over liposome-encapsulated antigens have become apparent: (i) A predominant coupling efficiency of antigens to liposomes; we have previously reported a procedure for coupling antigens to liposomes whereby approximately 50% of the antigens become bound to the surface of liposomes (11), whereas with antigen-encapsulation, a 60-fold greater volume of antigens is required to obtain the same amount of conjugates (unpublished observation). (ii) Antigen-specific and IgE-selective unresponsiveness induced by surface-linked liposomal antigens; antigens chemically coupled to the surface of liposomes induce antigen-specific IgG but not IgE antibody production in mice (10) and also in monkeys (39), suggesting that it might be possible to develop vaccines with minimal allergic side-effects by utilizing surface-linked liposomal antigens. In addition, during the course of an investigation intended to clarify the mechanism of IgE-selective unresponsiveness induced by surface-linked liposomal antigen, we identified an alternative mechanism, not involving T cells, in the regulation of IgE synthesis (13). (iii) Enhanced recognition of liposomal antigens by APCs; since liposomes basically consist of immunologically inert fatty acid, they are barely recognized by APCs. Therefore, some contrivance, such as the introduction of mannose on their surfaces (40), is required in antigen-encapsulated liposomes to enhance the recognition of liposomes by APCs. On the other hand, with surface-linked liposomal antigens, antigens expressed on the surface of liposomes might be recognized more efficiently by APCs, which could result in enhanced presentation to T cells. In fact, surface-linked liposomal antigens induce significantly greater antigen-specific IgG production than do liposome-encapsulated antigens in mice (unpublished observation). In addition, a significant difference has been observed between liposomes with different lipid components in the recognition of liposomal antigens by APCs, this difference correlating closely with the adjuvant activity of liposomes. More antigens coupled to "unsaturated" liposomes are engulfed by macrophages *in vitro*, and greater antigen-specific antibody production is induced *in vivo*, than when "saturated" liposomes are used, suggesting that the adjuvant effects of liposomes are exerted at the beginning of the immune response, that is, upon recognition of antigens by APCs (28). In addition to this quantitative difference between liposomes with different lipid components, a qualitative difference (i.e., a difference in ability to induce cross-presentation) has also been observed between "saturated" and "unsaturated" liposomes. Although the precise mechanism underlying this difference is currently unclear, the significant difference in membrane mobility observed between these liposomes (12) might affect their ability to induce cross-presentation. These data suggest that differences in lipid components in liposomes lead to differential processing and presentation of liposomal antigens in APCs.

Induction of antigen-specific $CD8^+$ T-cells and cytotoxic T lymphocytes by liposome-coupled cytotoxic T lymphocyte epitopes derived from internal proteins of influenza viruses

Based on the results described above, we evaluated liposomal conjugates with CTL epitope peptides derived from highly conserved internal antigens of influenza viruses for their ability to protect against infection by influenza viruses (41). HLA-A*0201 (A2)-binding epitopes were predicted among the amino-acid sequences of six coding regions—M1, NP, PA, PB1, PB2, and NS—in the H1N1 influenza virus (PR8 strain) using programs available on the Internet. The predicted epitopes were then

Table 1. Amino acid sequences of CTL epitopes derived from internal antigens of influenza viruses

Virus strain	M1 58–66	NS1 128–136	PB1 410–418
H1N1 (A/PR/8/34)	GILGFVFTL	IILKANFSV	GMFNMLSTV
H3N2 (A/Aichi/2/68)	GILGFVFTL	VMLKANFSV	GMFNMLSTV
H1N1 (A/New York/4290/2009)	GILGFVFTL	IVLKANFSV	GMFNMLSTV
H5N1 (A/Hong Kong/483/97)	GILGFVFTL	IILKANFSV	GMFNMLSTV

*Disagreements are underlined.

synthesized and chemically coupled to liposomes to evaluate their abilities to induce antigen-specific CD8$^+$ T-cells and CTLs by means of ICS and an *in vivo* cytotoxicity assay, respectively. Immunization of the liposome-coupled, HLA A2-restricted epitope peptides, M1 58–66, NS1 128–136, and PB1 410–418 (Table 1) induced significant numbers of antigen-specific CD8$^+$ T-cells in HLA-A2-transgenic (A2Tg) mice, as evaluated by ICS (Fig. 1a). Moreover, as shown in Figure 1b, all the peptide-liposome conjugates in Figure 1a induced significant *in vivo* CTL responses, indicating that all the predicted CTL epitopes were loaded onto MHC class I and recognized by CTLs. On the other hand, even in the presence of CpG solutions of the predicted peptides did not induce antigen-specific CD8$^+$ T-cells in A2Tg immunized mice (Fig. 1a). As shown in Table 1 all three peptides were preserved well, not only in H1N1 (A/PR/8/34) and H3N2 (A/Aichi/2/68) seasonal influenza viruses, but also in the 2009 pandemic H1N1 (A/New York/4290/2009) influenza virus and in a highly pathogenic H5N1 (A/Hong Kong/483/97) influenza virus In contrast, NS1 128–136 had partially changed in H3N2 (A/Aichi/2/68) and H1N1 (A/New York/4290/2009) influenza viruses. Among the CTL epitopes determined in the present study, peptide M1 58–66 had already been reported by Gotch *et al.* in 1987 (42).

Virus challenge experiment

We performed virus challenge experiments using peptide M1 58–66 on the above-described HLA-A2-restricted CTL epitopes. A2Tg mice were immunized subcutaneously with liposome-coupled, HLA-A2-restricted peptide M1 58–66. One week after the immunization, we infected them intranasally with H1N1 (A/PR/8/34) or H3N2 (A/Aichi/2/68) influenza virus. As shown in Figure 2, viral growth in the lung was suppressed significantly in the immunized mice 2 to 6 days after infection by either H1N1 (A) or H3N2 (B) influenza viruses. Thus, in A2Tg mice, immunization with liposome-coupled peptides successfully induced protection against influenza viruses regardless of the influenza virus subtypes.

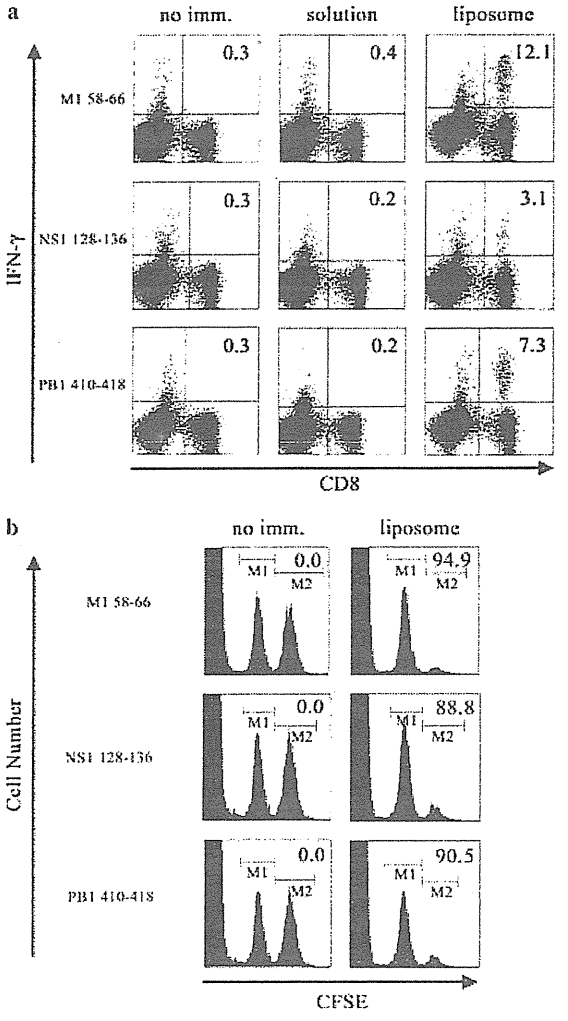

Fig. 1. Induction of antigen-specific CD8$^+$ T-cell and CTL responses. Mice received immunization with the liposome-coupled peptides M1 58–66, NS1 128–136, and PB1 410–418 (liposome) or liquefied peptides in the same amounts as liposome conjugates (solution) in the presence of CpG. Control mice received no immunization. One week after immunization ICS and *in vivo* CTL assay were performed. (a) ICS of antigen-specific CD8$^+$ T-cells among spleen cells of A2Tg mice. Cells were stained for their surface expression of CD8 (x axis) and their intracellular expression of IFN-γ (y axis). The numbers shown indicate the percentage of CD8$^+$ cells that were positive for intracellular IFN-γ. (b) Induction of CTLs by liposome-coupled peptides. The numbers shown indicate the percentage of total cells killed. The data shown are representative of three independent experiments. Imm., immunization.

Induction of memory CD8$^+$ T-cells

After confirming induction of protective ability by liposomal peptide in the effector phase, we investigated whether or not this immunization induced memory CD8$^+$ T-cells. As shown in Figure 3, significant numbers of CD8$^+$ T-cells

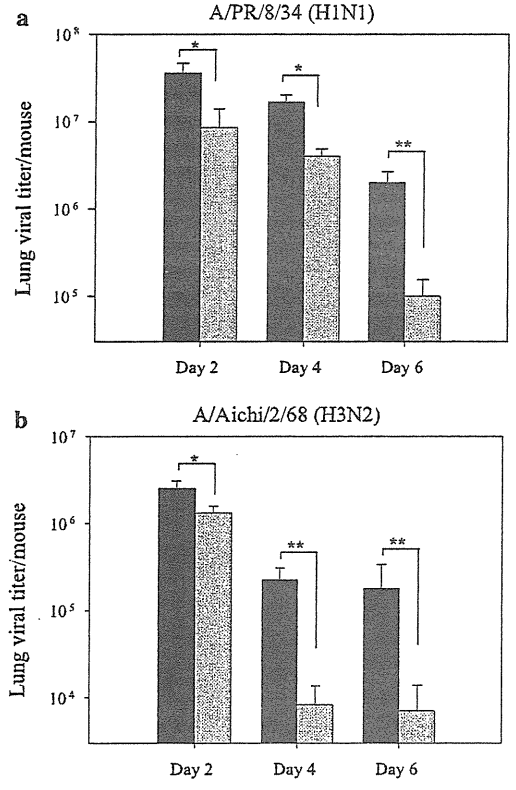

Fig. 2. Viral titers in lungs of mice following challenge with H1N1 or H3N2 influenza virus. Mice received immunization with either liposome-coupled peptide M1 58–66 liposome conjugates in the presence of CpG (gray bars) or none (black bars). One week later, mice were challenged intranasally with either (a) the H1N1 or (b) the H3N2 influenza virus. On days 2, 4, or 6 post-infection, viral titers in the lungs were determined by calculating the 50% tissue culture infective dose using MDCK cells. Data represent mean and standard error of five mice per group. *, $P < 0.05$; **, $P < 0.01$.

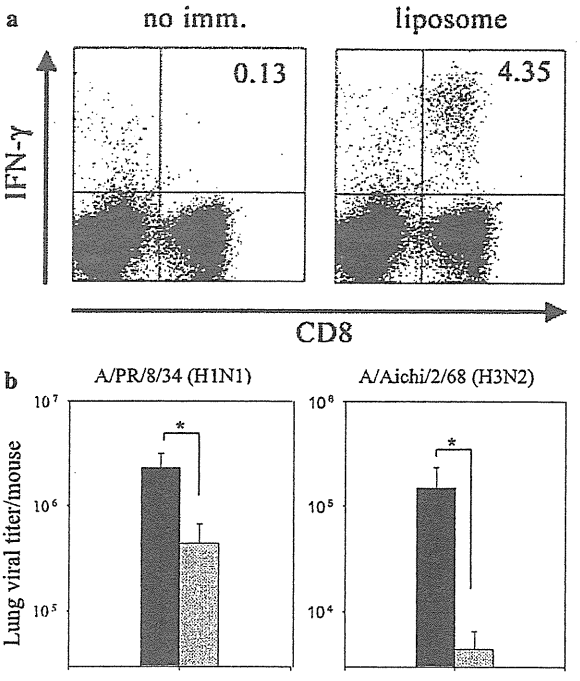

Fig. 3. Induction of long-lasting immunity by peptide-liposome conjugates. Mice were immunized with liposome-coupled peptide M1 58–66 either in the presence or absence of CpG. 90 days after immunization, ICS and viral challenge experiments were performed. (a) ICS of $CD8^+$ T-cells specific for peptide M1 58–66 among spleen cells of A2Tg mice immunized with liposome-coupled peptide (liposome) or not (no imm.). (b) Viral replication in the lungs of mice immunized with liposome-coupled peptide (gray bars) or not (black bars) 6 days after nasal infection with influenza viruses. Data represent the mean and standard error of five mice per group. imm., immunization; *, $P < 0.05$.

specific to peptides M1 58–66 were detected in the immunized mice at 90 days after immunization (Fig. 3a). In addition, viral growth in the lung was significantly suppressed after nasal challenge with either the H1N1 or H3N2 influenza virus (Fig. 3b). Thus, we demonstrated that immunization readily induces memory $CD8^+$ T-cells.

Induction of long-lasting protection against lethal doses of influenza viruses

We next investigated whether or not the long-lasting immunity demonstrated above would help protect mice against infection with lethal doses of influenza viruses. Six months after immunization with liposome-coupled M1 58–66 peptides, mice were challenged with a lethal dose of influenza virus H1N1 PR8 strain. As shown in Figure 4, although the immunized mice lost body weight up to 8 days after infection, at 4 to 7 days the rate of loss was significantly less than that observed in a non-immunized control group, and the immunized mice recovered body weight thereafter (Fig. 4a). Finally, five out of six immunized mice survived, whereas all of the non-immunized control mice died within 7 days of infection with significant loss of body weight and morbidity (Fig. 4b).

The data cited above indicate that liposome-coupled CTL epitope peptides derived from internal antigens of influenza virus protect against infection with heterosubtypic influenza viruses. Since the early finding that influenza A virus-specific CTLs are broadly cross-reactive for cells of the same MHC class I type infected with serologically distinct H1N1 and H3N2 viruses (20), numerous investigators have reported on immunodominant and cross-reactive CTL epitopes derived from conserved internal antigens, such as NP (43, 44), M1 (42, 44, 45), and NS1 (21) influenza viruses. It was hoped that CTL epitopes could be used to develop a broadly protective

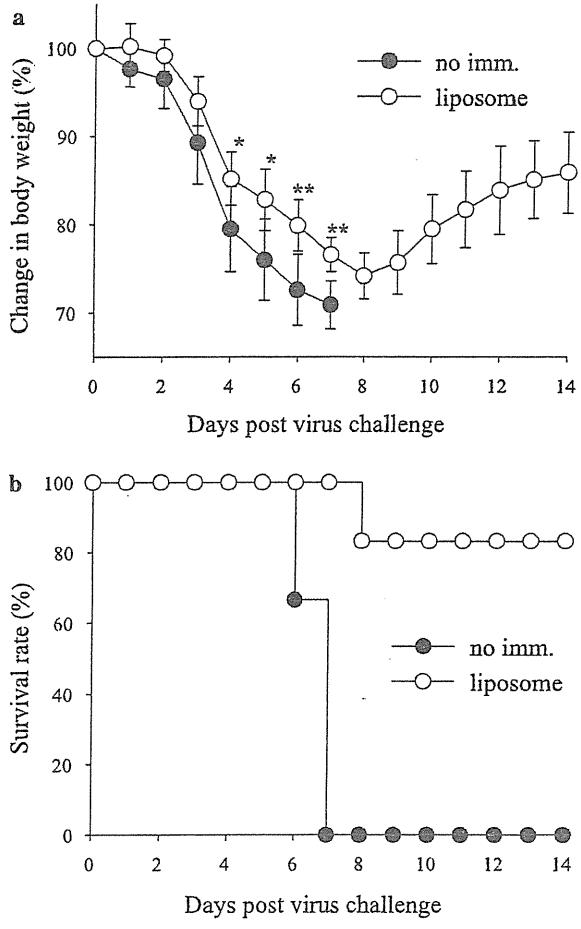

Fig. 4. Maintenance of protective ability against a lethal dose of H1N1 virus in mice immunized with liposome-coupled peptide. Mice were immunized with either liposome-coupled peptide M1 58–66 in the presence (open symbols) or absence (solid symbols) of CpG. Six months after immunization, mice were challenged intranasally with 5 × median lethal dose of H1N1 influenza virus (PR8 strain). The mice were weighed and monitored daily for mortality up to 14 days post-challenge. (a) Changes in body weight of mice calculated as a percentage of the mean weight per group in comparison with starting body weight. Data represent mean body weight and standard error of six mice per group. *, $P < 0.05$: **, $P < 0.01$ as compared with non-immunized control mice. (b) Survival percentage. The mice were killed once they had lost 30% of their initial body weight.

influenza vaccine (46). To date, candidate $CD8^+$ T-cell vaccines have been reported to induce partial protection (47), suggesting that $CD8^+$ T-cells certainly help to protect against influenza. Therefore, the $CD8^+$ T-cell vaccine strategy against influenza was expected to support antibody-focused vaccine strategies by "reducing" (46) or "dampening" (21) the impact of the next pandemic.

Liposome-coupled CTL epitope peptides efficiently induced antigen-specific $CD8^+$ T-cells and CTLs (Fig. 1), and suppressed viral replication in the lungs of mice infected with either H1N1 or H3N2 influenza virus (Fig. 2). In addition, since a single immunization just one week before infection successfully reduced viral replication in the lung, this vaccination protocol would be expected to counter the rapid spread of an influenza pandemic. The efficacy of liposome-coupled peptides in inducing CTL-based protective immunity is likely due to the characteristics of liposome-coupled antigens, which are very readily recognized by APCs (28) and effectively induce cross-presentation via MHC class I in APCs (16) In addition to their safety, they are less likely to induce allergic responses (13). Moreover, vaccines consisting of liposome-coupled CTL epitope peptides without $CD4^+$ T-cell epitopes are capable of inducing long-lived $CD8^+$ memory T-cells (Fig. 3). Mice immunized with liposome-coupled M1 58–66 peptides remained protected for at least 6 months after immunization (Fig. 4).

The CTL epitopes employed in this study are contained not only in the seasonal influenza viruses, but also in the currently emerging S-OIV and in the extremely virulent avian H5N1 influenza viruses (Table 1), suggesting that this liposomal peptide vaccine might be effective in protecting against infection by both seasonal and pandemic influenza viruses. In addition, the liposome employed in this study was originally developed as an antigenic carrier that effectively induces humoral immunity (i.e., antibody production) against liposome-coupled antigens (14). Therefore, antigen-liposome conjugates are capable of inducing both humoral and cellular immunity against influenza viruses, by combined coupling of antibody- and CTL epitopes to the surfaces of liposomes.

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Liposome-Coupled Peptides Induce Long-Lived Memory CD8$^+$ T Cells Without CD4$^+$ T Cells

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Abstract

CD8$^+$ T cells provide broad immunity to viruses, because they are able to recognize all types of viral proteins. Therefore, the development of vaccines capable of inducing long-lived memory CD8$^+$ T cells is desired to prevent diseases, especially those for which no vaccines currently exist. However, in designing CD8$^+$ T cell vaccines, the role of CD4$^+$ T cells in the induction and maintenance of memory CD8$^+$ T cells remains uncertain. In the present study, the necessity or not of CD4$^+$ T cells in the induction and maintenance of memory CD8$^+$ T cells was investigated in mice immunized with liposome-coupled CTL epitope peptides. When OVA-derived CTL epitope peptides were chemically coupled to the surfaces of liposomes and inoculated into mice, both primary and secondary CTL responses were successfully induced. The results were further confirmed in CD4$^+$ T cell-eliminated mice, suggesting that CD4$^+$ T cells were not required for the generation of memory CD8$^+$ T cells in the case of immunization with liposome-coupled peptides. Thus, surface-linked liposomal antigens, capable of inducing long-lived memory CD8$^+$ T cells without the contribution of CD4$^+$ T cells, might be applicable for the development of vaccines to prevent viral infection, especially for those viruses that evade humoral immunity by varying their surface proteins, such as influenza viruses, HIV, HCV, SARS coronaviruses, and Ebola viruses.

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Introduction

It has been reported by numerous investigators that CD4$^+$ T cells are essential for the maintenance of memory CD8$^+$ T cells [1–5]. However, in the induction and maintenance of CD8$^+$ memory T cells, different roles of CD4$^+$ T cells have been described [6–9]. In the so-called "classical model", CD4$^+$ T cells contribute to memory CD8$^+$ T-cell generation indirectly via APCs [6]. Through the CD40-CD40L interaction between CD40L on CD4$^+$ T cells and CD40 on APCs, CD4$^+$ T cells "license" APCs for the induction of memory CD8$^+$ T cells. As an alternative to this APC licensing model, Bourgeois et al. [7] provided evidence demonstrating that CD4$^+$ T cells contribute directly to CD8$^+$ T cells through CD40 on CD8$^+$ T cells, rather than indirectly via APCs. However, these findings were countered by studies in which long-lived CD8$^+$ memory T cells were generated in the absence of CD40 expression on CD8$^+$ T cells [8,9]. In addition, as for the role of CD40-CD40L interaction in the induction of memory CD8$^+$ T cells, Hernandez et al. [10] reported that CD8$^+$ T cells themselves provided CD40L in order to license APCs for the induction of memory CD8$^+$ T cells. In their scenario, although the CD40-CD40L interaction between T cells and DCs is indispensable for the induction of memory CD8$^+$ T cells, CD4$^+$ T cells are not necessarily involved. Thus, the research so far has not resolved the role of CD4$^+$ T cells in the induction and maintenance of memory CD8$^+$ T cells, although resolving this issue is a critical step in designing better vaccination and immunotherapeutic strategies.

Upon natural infection, the host responds by inducing humoral and cellular immunity against the pathogen. Humoral immune responses are represented by the production of antibodies that bind to the surfaces of bacteria and viruses, whereas cellular immune responses mediate immunity to intracellular pathogens. In general, extracellular antigens are presented via MHC class II molecules to CD4$^+$ T cells, whereas intracellular antigens are presented via MHC class I molecules to CD8$^+$ T cells. To induce antigen-specific CTL, antigens must be loaded onto the class I MHC processing pathway in APCs via cross-presentation [11]. In the cross-presentation, exogenous proteins cross over to the endogenous pathway to gain access to MHC class I molecules. Using this phenomenon, a generation of antigen-specific CTL responses might be useful in the development of vaccines that can prevent viral diseases. However, the currently approved alum adjuvant, which was first described by Glenny et al. [12] in 1926 and until today remains the only adjuvant approved for clinical use, is known to be effective only for the induction of humoral immunity, not for the induction of cell-mediated immunity [13–16]. Consequently, the development of a novel vaccine adjuvant is essential for the induction of cell-mediated immunity.

We previously reported that surface-coupled liposomal antigens could be presented by APCs to CD8$^+$ T cells via MHC class I molecules if certain lipid components were chosen for the liposomes [17]. This antigen preparation was expected to be applicable for the development of tumor vaccines to induce antitumor responses and for the development of viral vaccines to

induce virus-specific CTLs that effectively eliminate virus-infected cells [18]. Since the liposomal conjugates induced CTLs efficiently when CTL epitope peptides were coupled to the surfaces of liposomes [17], the liposomal conjugatesare expected to be applicable for the development of CTL-based peptide vaccines. In the development of peptide vaccines, it is essential to know whether a T helper epitope peptide is necessary for the induction of long-lived memory $CD8^+$ T cells, an important step in vaccine preparation. This study was aimed at evaluating the role of $CD4^+$ T cells in the induction of long-lived memory $CD8^+$ T cells by liposome-coupled peptides.

Results

Induction of antigen-specific primary $CD8^+$ T cells and CTLs in mice by $OVA_{257-264}$-liposome conjugates

Mice were immunized with $OVA_{257-264}$-liposome conjugates in the presence of CpG as described in Materials and Methods. A significant induction of CTL specific for $OVA_{257-264}$ was observed on day 4 and a complete cell killing was observed as early as 5 days after the immunization (Figure 1). Therefore, in the following experiments, primary CTL responses were monitored at 7 days after immunization. Mice were then immunized with serially diluted solution of $OVA_{257-264}$-liposome conjugates containing 0.3 (8×) to 2.4 μg (1×) of peptides or $OVA_{257-264}$ solution that contained equal amounts of peptides as those in liposomal conjugates. Although $OVA_{257-264}$-liposome and $OVA_{257-264}$ solution seemed to induce a comparable level of T-cell cytokine production at the highest dose (2.4 μg/injection), a dose-dependent decrease was observed in mice immunized with $OVA_{257-264}$ solution but not in mice immunized with $OVA_{257-264}$-liposome, suggesting that $OVA_{257-264}$-liposome was more effective than $OVA_{257-264}$ solution in the induction of antigen-specific $CD8^+$ T cell cytokine production (Figure 2A). Similar results were observed in T cell cytokine production; a dose of $OVA_{257-264}$-liposome as low as 0.6 μg/mouse (4× dilution) induced a perfect killing as assayed by *in vivo* CTL assay, while $OVA_{257-264}$ solution induced only a partial killing even at the highest dose (Figure 2B).

Secondary CTL response in mice immunized with $OVA_{257-264}$-liposome

Induction of secondary CTL responses in mice immunized with $OVA_{257-264}$-liposome was further investigated. Mice were immunized with 50 μl of $OVA_{257-264}$-liposome and 2, 4, 8, 16, and 20

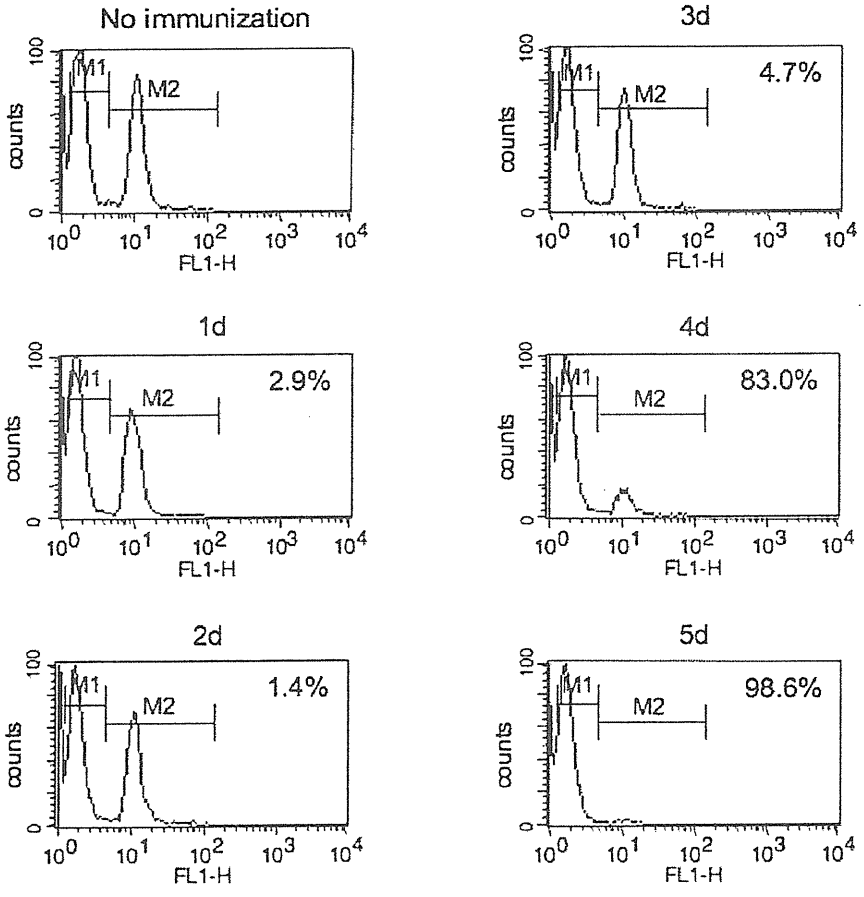

Figure 1. Kinetics of primary CTL response induced by $OVA_{257-264}$-liposome conjugates. Mice were immunized with 50 μl of $OVA_{257-264}$-liposome in the presence of 5 μg CpG; one to 5 days later, an *in vivo* CTL assay was performed as described in Materials and Methods. The numbers for each time period indicate percentages of target cells killed. Data are representative of three individual mice in each group for which similar results were obtained.
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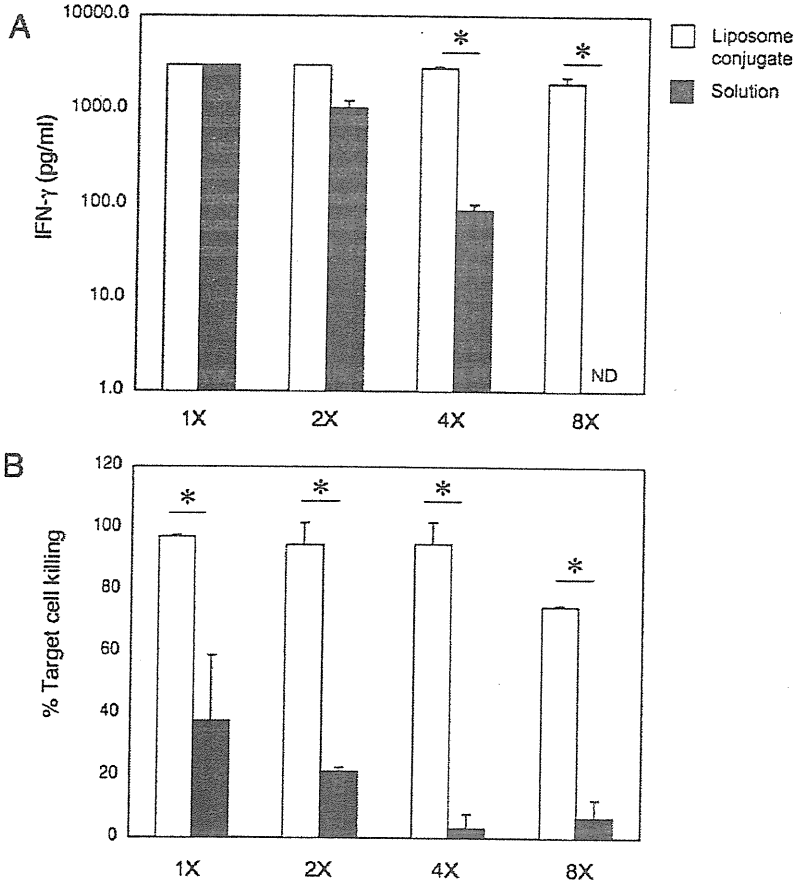

Figure 2. Dose-response of cytokine production by $CD8^+$ T cell and CTL induction in mice immunized with $OVA_{257-264}$-liposome or with $OVA_{257-264}$ solution. A serial two-fold dilution of $OVA_{257-264}$-liposome (open box) and $OVA_{257-264}$ solution (closed box) were made in PBS, and mice were immunized with the diluents in the presence of 5 μg CpG. $OVA_{257-264}$ solution containing equal amounts of peptides as those in $OVA_{257-264}$-liposome. One week after the immunization, IFN-γ production by $CD8^+$ T cells (**A**) and the CTL response (**B**) were monitored as described in Materials and Methods. Data represent means and SE of three mice per group. *, significant difference ($p>0.01$).
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weeks later, the mice received a booster injection with OVA. Three days after the booster injection, $OVA_{257-264}$-specific cell killing was monitored. As shown in Figure 3, a complete cell killing was observed at 2 weeks after the immunization without a booster injection and, up to 20 weeks after the immunization, a significant recall response was observed upon booster injection with OVA. Inoculation of naive mice with the same dose of OVA as the booster injection ("No imm." in Figure 3) did not induce a detectable CTL response. Interestingly, a significant recall response was observed even at 20 weeks when the primary CTL response was nearly undetectable. An antigen-specific $CD8^+$ T-cell proliferation assay further confirmed the results; as shown in Figure 4, $CD8^+$ T cells of mice immunized with $OVA_{257-264}$-liposome significantly proliferated upon *in vitro* stimulation with OVA even 20 weeks after immunization.

Effect of in vivo elimination with $CD4^+$ T cells on the induction of long-lived memory $CD8^+$ T cells by $OVA_{257-264}$-liposome conjugates

To eliminate $CD4^+$ T cells, mice were inoculated with GK1.5 as described in Materials and Methods, and immunized with $OVA_{257-264}$-liposome. As shown in Figure 5, *in vivo* elimination with $CD4^+$ T cells affected neither for primary (Figure 5A) nor for secondary (Figure 5B) CTL responses; even at 20 weeks after the immunization, a significant recall response, comparable to that in normal mice, was observed in mice from which $CD4^+$ T cells had been eliminated.

Discussion

In the present study, the role of $CD4^+$ T cells in the induction and maintenance of memory $CD8^+$ T cells was evaluated in mice immunized with liposome-coupled CTL epitope peptides. Although the inclusion of CpG, a ligand of TLR-9, was needed for the induction of the primary CTL response by $OVA_{257-264}$-liposome, $CD4^+$ T cells were not required in either primary or secondary response, since long-lived memory $CD8^+$ T cells were readily induced only by immunization with CTL epitope peptides coupled to liposomes (Figures 3 and 4). This finding was further confirmed in $CD4^+$ T cell-depleted mice (Figure 5). These results are in agreement with those reported previously by numerous investigators that $CD4^+$ T cells are dispensable for the primary expansion of $CD8^+$ T cells and their differentiation into cytotoxic

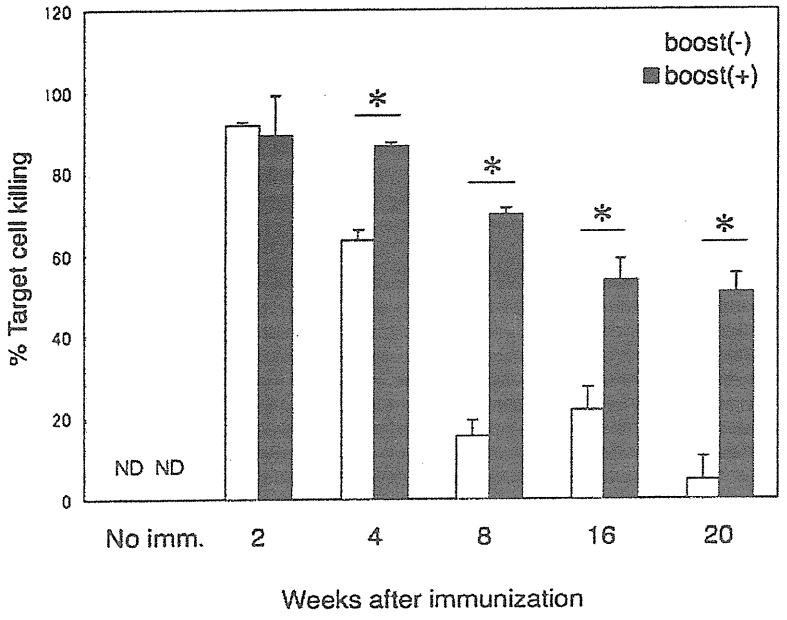

Figure 3. Secondary CTL response in mice immunized with $OVA_{257-264}$-liposome. Mice were immunized with 50 μl of $OVA_{257-264}$-liposome in the presence of 5 μg CpG, and 2, 4, 8, 16, and 20 weeks later, they received a booster ip injection with 200 μl of 1 mg/ml OVA in PBS (closed box) or no booster injection (open box). Three days after the booster injection, *in vivo* CTL assay was performed. Data represent mean percentages of cells killed and SEs of three mice per group. ND, not detected. *, significant difference ($p>0.01$).
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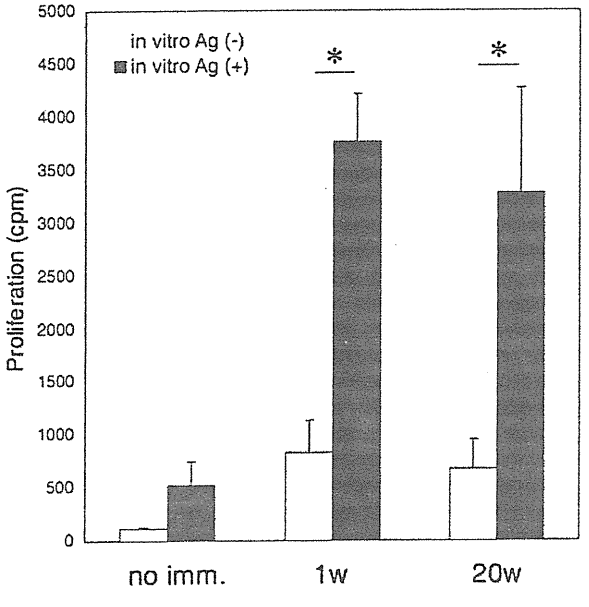

Figure 4. Antigen-specific $CD8^+$ T-cell proliferation assay. Mice were immunized with $OVA_{257-264}$-liposome and 1 week or 20 weeks later, $CD8^+$ T cells of the immunized mice were cultured in the presence (closed box) or absence (open box) of OVA as described in Materials and Methods. Data represents mean ^3H- thymidine incorporation and SE of triplicate cultures. *, significant difference ($p>0.01$).
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effectors [2,3,5]. However, most of these researchers have claimed that secondary CTL expansion is wholly dependent on the presence of T helper cells during, but not after, priming [1–5].

We previously reported that surface-linked liposomal antigens induced IgE-selective unresponsiveness [19]. The results were consistent even when different coupling procedures for the antigens with the liposomes were employed [20]. During the course of an investigation intended to clarify the mechanism of IgE-selective unresponsiveness induced by surface-coupled liposomal antigens, we discovered an alternative approach to regulating the production of IgE, one that is independent of the activity of T cells [21]. Immunization of mice with OVA-liposome conjugates induced IgE-selective unresponsiveness without apparent Th1 polarization. Neither interleukin-12 (IL-12), IL-10, nor $CD8^+$ T cells participated in the regulation. Further, $CD4^+$ T cells of mice immunized with OVA-liposome were capable of inducing antigen-specific IgE synthesis in athymic nude mice immunized with alum-adsorbed OVA. On the other hand, immunization of the recipient mice with OVA-liposome did not induce anti-OVA IgE production, even when $CD4^+$ T cells of mice immunized with alum-adsorbed OVA were transferred. In the secondary immune response, OVA-liposomes enhanced anti-OVA IgG antibody production but did not enhance ongoing IgE production, suggesting that the IgE-selective unresponsiveness induced by the liposomal antigen involved direct effects on IgE but not IgG switching *in vivo*. These results suggest the role of an alternative mechanism, one not involving T cells, in the regulation of IgE synthesis, and raise the possibility that the surface-linked liposomal antigens are potentially applicable for the development of novel vaccines with minimal induction of IgE synthesis. Moreover, given the relatively low allergic response to and increased antigenicity of the allergen, this form of antigen preparation would be applicable for allergen immunotherapy [22].