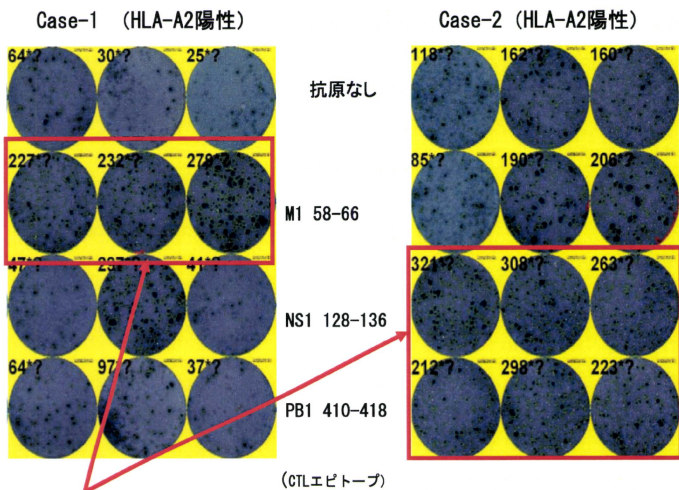


図-1： これまでに同定されたヒトHLA拘束性CTLエピトープ
 に対するヒト末梢血単核球の反応性



* 有意差あり

厚生労働科学研究費補助金（医療機器開発推進研究事業）
分担研究報告書

インフルエンザウイルス抗原に対する CTL 応答のヒトにおける解析

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研究要旨

ヒトにおけるインフルエンザウイルス抗原に対する CTL 応答を解析するために、慈恵医大教職員および学生を対象としたアンケート調査により、インフルエンザの流行状況を把握するとともに細胞性免疫がどのくらい誘導されているのか解析を試みた。平成 22 年 2 月～6 月のアンケート調査（回答数 1,089 名）から、平成 21 年度の発熱既往およびインフルエンザ迅速診断キット陽性率は学生群（平均年齢 21.3 歳）でそれぞれ 60%、40% ともっとも高く、学生間で新型インフルエンザの爆発的な流行があったと推測される。これに対して、教職員のそれらは学生に比べて有意に低く年齢が高くなるにつれて低下する傾向にあった。一方、平成 23 年 2 月～3 月の学生を対象としたアンケート調査では、平成 22 年度の罹患率は 5 分の 1 以下であった。なお、インフルエンザ罹患率とワクチン接種との間に有意な相関は認められなかった。インフルエンザウイルス抗原に対する CD8 陽性 T 細胞の反応を、同定した HLA-A2 および A24 拘束性 CTL エピトープペプチドを抗原として用い、末梢血単核球の増殖によって解析した。その結果、HLA-A2 拘束性の M1 由来ペプチドに対する CD8 陽性 T 細胞の反応は、学生群（平均年齢 20.8 ± 1.7、刺激指数は 2.18 ± 0.78）が教職員（平均年齢 42.3 ± 10.3、刺激指数 1.63 ± 0.65）に比較して有意に高かった。また、HLA-A24 拘束性 CTL エピトープペプチドに関しても、反応性は低い学生群で CD8 陽性 T 細胞の反応が高い傾向にあった。これらの結果は、インフルエンザウイルス感染防御に細胞性免疫応答が関与していることを示唆している。

A. 研究目的

インフルエンザ感染を防御する免疫機構には、抗体による液性免疫と細胞傷害性 T 細胞（CTL）による細胞性免疫とがあることが知られている。今回、ヒトにおけるインフルエンザウイルス抗原に対する細胞性免疫（CD8 陽性 T 細胞）応答を解析するために、アンケート調査によるインフルエンザの罹患状況と末梢血単核球を用いた CD8 陽性 T 細胞応答

の解析を試みた。

B. 研究方法

対象は本学教職員および学生とした。平成 22 年 2 月～6 月および平成 23 年 2 月～3 月（学生のみ対象）にアンケート用紙を配布し得られた回答について解析を行った。細胞性免疫は、同意の得られた被験者から採血し、同定したインフルエンザウイルス由来、HLA-A2 拘束性 CTL エピトープペプチド 3 種類と

HLA-A24 拘束性 CTL エピトープペプチド 5 種類を抗原として用い、末梢血単核球由来 CD8 陽性 T 細胞の増殖反応によって解析した。

C. 研究結果

平成 22 年 2 月～6 月のアンケート調査で回答が得られた人数は 1,089 名であった。内訳は学生 362 名、教職員 662 名、年齢性別不詳 65 名である。そこで、調査対象者を学生（平均年齢：21.3）と、教職員を 21～30 歳 154 名（26.99）、31～40 歳 222 名（35.65）、41～50 歳 164 名（45.30）と 51 歳以上 122 名（57.32）の年齢別に分けて解析を行った。この 1 年間（平成 21 年度）の発熱の既往およびインフルエンザ迅速診断キット陽性率は学生群でもっとも高く、それぞれ 60%、43% であり、年齢が高くなるにつれて低下した。若年齢においてはインフルエンザに罹患して発熱しやすいことが示唆された。一方、平成 23 年 2 月～3 月の学生のみ（回答数 377 名）を対象とした調査では、発熱の既往：11.1%、インフルエンザ迅速診断キット陽性率：9.5% と、平成 22 年度のインフルエンザ感染による発症率は平成 21 年度と比較して極めて低いことが判明した。

末梢血単核球を用いた HLA-A2 拘束性ペプチド 3 種類および HLA-A24 拘束性ペプチド 5 種類に対する CD8 陽性 T 細胞の反応は、HLA-A2 拘束性のペプチド 3 種類に対して高い増殖反応が認められた。特に HLA-A2 拘束性の M1 由来ペプチドに対する CD8 陽性 T 細胞の反応は、学生群（平均年齢 20.8±1.7）の刺激指数が 2.18±0.78 と、教職員（平均年齢 42.3±10.3）の刺激指数 1.63±0.65 と比較して有意に高かった。また、HLA-A24 拘束性ペプチド 5 種類に対しても、反応性は低

いが学生群で CD8 陽性 T 細胞の反応が高い傾向にあった。

D. 考察

アンケート調査により、平成 21 年度は学生間で新型インフルエンザの爆発的な流行があったことが推測される。学生において CD8 陽性 T 細胞の反応が高いのは、インフルエンザに感染した結果として細胞性免疫が誘導されたためと考えられる。これに対して平成 22 年度のインフルエンザ罹患率が極めて低かったのは、平成 21 年度のインフルエンザ感染によって液性および細胞性免疫が誘導された結果であると推測される。

E. 研究発表

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該当無し

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G. 知的財産権の出願・登録状況（予定を含む。）

1. 特許出願

該当無し

2. 実用新案登録

該当無し

3. その他

該当無し

Ⅲ. 研究成果の刊行に関する一覧表

書籍

該当なし

雑誌

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IV. 研究成果の刊行物・別刷

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

内田哲也

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

1 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性が生じる。

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

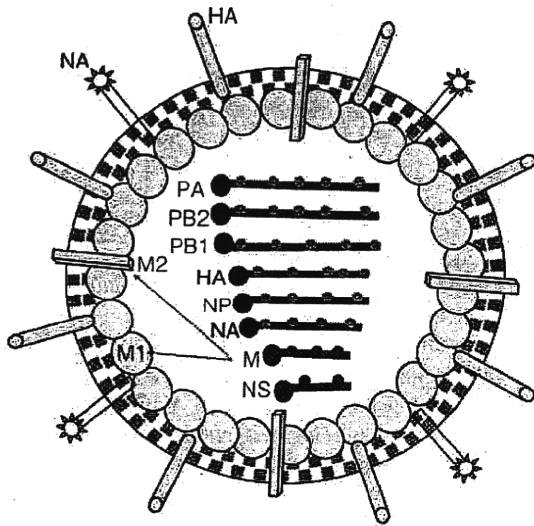


図1 インフルエンザウイルスの構造

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 cytotoxic 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 lu-

men 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 OVA₂₅₇₋₂₆₄ 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 in-

duced 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	GMFNMMLSTV
H3N2 (A/Aichi/2/68)	GILGFVFTL	<u>V</u> MLKANFSV	GMFNMMLSTV
H1N1 (A/New York/4290/2009)	GILGFVFTL	<u>I</u> VLKANFSV	GMFNMMLSTV
H5N1 (A/Hong Kong/483/97)	GILGFVFTL	IILKANFSV	GMFNMMLSTV

*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.

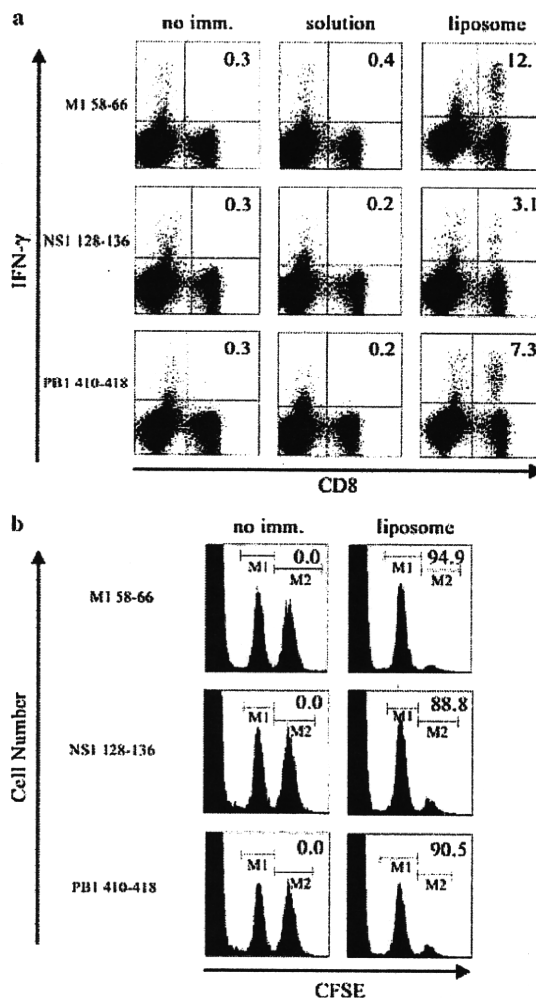


Fig. 1. Induction of antigen-specific CD8⁺ T-cell and CTL responses. Mice received immunization with the liposome-coupled peptides M1 58–66, NS1 128–136, and PB1 410–418 (liposome) or liquefied peptides in the same amounts as liposome conjugates (solution) in the presence of CpG. Control mice received 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

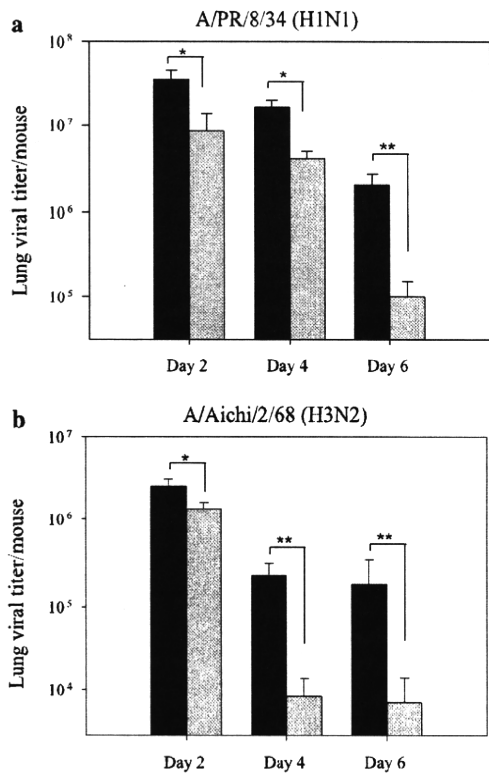


Fig. 2. Viral titers in lungs of mice following challenge with H1N1 or H3N2 influenza virus. Mice received immunization with either liposome-coupled peptide M1 58–66 liposome conjugates in the presence of CpG (gray bars) or none (black bars). One week later, mice were challenged intranasally with either (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. *, $P < 0.05$; **, $P < 0.01$.

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

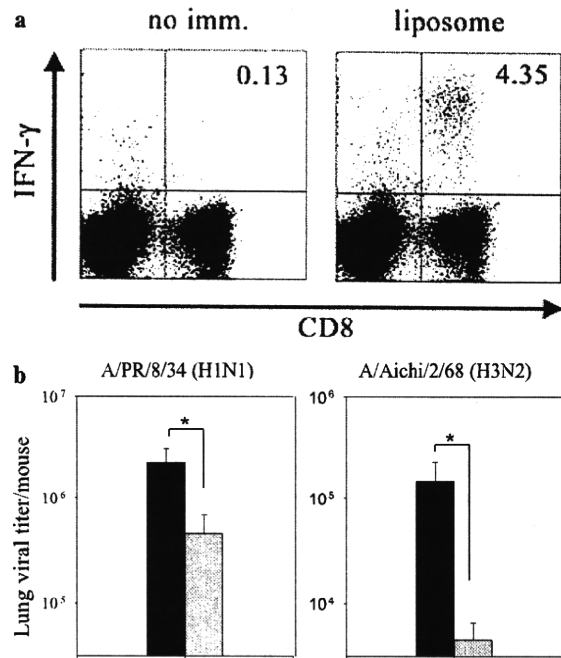


Fig. 3. Induction of long-lasting immunity by peptide-liposome conjugates. Mice were immunized with liposome-coupled peptide M1 58–66 either in the presence or absence of CpG. 90 days after immunization, ICS and viral challenge experiments were performed. (a) ICS of CD8⁺ T-cells specific for peptide M1 58–66 among spleen cells of A2G 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; *, $P < 0.05$.

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

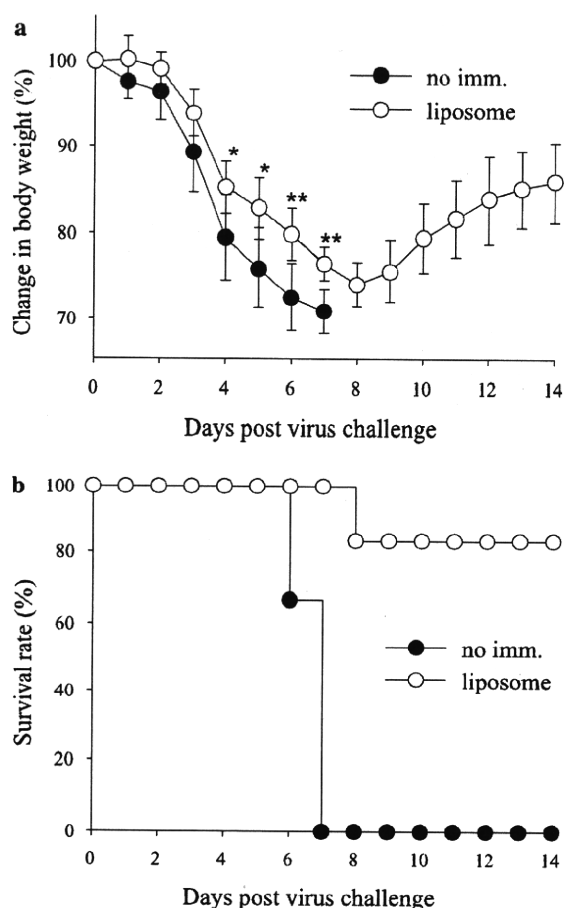


Fig. 4. Maintenance of protective ability against a lethal dose of H1N1 virus in mice immunized with liposome-coupled peptide. Mice were immunized with either liposome-coupled peptide M1 58–66 in the presence (open symbols) or absence (solid symbols) of CpG. Six months after immunization, mice were challenged intranasally with $5 \times$ 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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