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Advances in Brief

The Induction of Cytotoxic T Cells and Tumor Regression by Soluble Antigen Formulation

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Abstract

CTLs specific for tumor antigens play a major role in the immunity against cancer. We have shown that class I-restricted CTLs can be induced by injecting soluble antigens mixed in an antigen formulation (AF) that consists of squalane, Tween 80, and Pluronic L121 (S. Raychaudhuri *et al.*, Proc. Natl. Acad. Sci. USA, *89:* 8308–8312, 1992). In this study, using ovalbumin and the ovalbumin-expressing transfectoma (EG7) as a tumor model system, we examined the *in vivo* antitumor effect of antigen-AF mixture. Vaccination of mice with ovalbumin in AF 2 or 3 days after EG7 tumor challenge showed significant inhibition of tumor growth compared to mice vaccinated with ovalbumin in alum or in saline. Depletion of $CD8^+$ cells at the time of immunization completely abrogated the AF-induced tumor protection, indicating that $CD8^+$ T cells are the major effectors in tumor protection *in vivo*. Depletion of $CD4^+$ cells led to a marginal loss of tumor protection, which may be the result of inhibition of ovalbumin-specific CTL response due to the lack of T-helper activity. Our results demonstrate that AF can be used in subunit vaccines to stimulate CTLs and tumor regression *in vivo*.

Introduction

Induction of $CD8^+$ CTLs specific for tumor antigens provides an attractive basis for the generation of new cancer vaccines (1–3). Although a variety of host immune effector cells have been shown to participate in the killing of tumor cells, tumor-specific CTLs are highly specific and effective in mediating tumor killing, even at low antigen density expressed on the target cells (2, 3). CTLs eliminate tumor cells by recognizing antigenic peptides in association with MHC class I molecules on the surface of tumor cells (2, 4, 5). Presentation of antigens by class I or class II molecules depends on the outcome of intracellular processing pathways. Exogenous, nonreplicating agents and soluble antigens enter the endosomal pathway and are presented at the cell surface in association with class II molecules, activating $CD4^+$ T cells. Alternatively, endogenously synthesized antigens such as tumor or viral antigens enter a different pathway to associate with class I molecules and activate $CD8^+$ CTLs (5, 6). Immunization with purified soluble tumor antigens or tumor cell lysates alone have resulted in ineffective CTL responses (7), suggesting that replicating vector systems or new formulations are needed for the incorporation of these antigens into the class I pathway. Compelling evidence from the ovalbumin system indicated that it is possible to force exogenous antigen into the cytosolic class I pathway and induce CTL response (8, 9). In these studies, immunization of mice with syngeneic splenocytes loaded with ovalbumin by osmotic lysis of pinosomes has led to the generation of class I-restricted $CD8^+$ CTL response. Based on this principle, different strategies involving proteins and peptides conjugated to lipophilic compounds or formulated with immune stimulating complexes or pH-sensitive liposomes have been used to elicit antigen-specific $CD8^+$ CTL response (10–13).

Recently, we have described a chemically defined AF[3] composed of squalane, Tween 80, and Pluronic L121 and demonstrated that soluble antigens injected with AF induce antigen-specific $CD8^+$ class I-restricted CTL responses (14). In this study, using an ovalbumin-expressing EL-4 (EG7) tumor model, we have attempted to elucidate the significance of ovalbumin/AF-induced immune response *in vivo* for tumor regression.

Materials and Methods

Animals. Female C57BL/6 (H-2^b) mice, ages 6–8 weeks, were obtained from Harlan Sprague-Dawley. Animal studies were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" specified by the Committee on Care of Laboratory Animals Resources Commission on Life Science-National Research Council (15).

Reagents. Grade VII Chicken ovalbumin and complete Freund's adjuvant were purchased from Sigma Chemical Co. Alum was purchased from Pierce Chemical Co. The anti-CD4 mAb RL.172 and the anti-CD8 mAb 3.168 were kind gifts from Dr. J. Sprent (The Scripps Clinic and Research Foundation, La Jolla, San Diego; Ref. 14).

Cell Lines. Ia^- thymoma cell line EL4 (C57BL/6, H-2^b) and a transfectoma derived from EL4-expressing chicken ovalbumin, EG7 (9), were kindly provided by Dr. Michael J. Bevan (University of Seattle, Seattle, WA). EG7 cells were propagated *in vitro*, as described elsewhere (15).

Preparation of AF. The components squalane (Aldrich), Tween 80 (Aldrich), and Pluronic L121 (BASF, Parisppany, NJ) were dissolved in PBS (pH 7.4) to form an oil-in-water emulsion comprising 15% (w/v) squalane and 0.6% Tween 80 with 3.75% Pluronic L121. The emulsion was then cycled through a M110T microfluidizer (Microfluidics, Newton, MA) six times at 4°C to obtain a stable homogenous emulsion, with a mean particle size ranging from 50 to 300 nm. The resultant emulsion was sterile filtered by using a 0.2 μm filter and stored under nitrogen before use. The emulsion was tested for the presence of endotoxin by *Limulus* amebocyte-lysate gel-clot assay. Adjuvants were stored at 4°C for 1 year without any effects on its stability to stay as a homogenous emulsion or on its adjuvancity.

Immunization. Mice were injected s.c. with a native form of ovalbumin in adjuvants in a total volume of 200 μl/mouse. Before immunization, triple-strength AF was diluted by mixing with 30 μg ovalbumin in HBSS to the final volume and mixed for 60 s. This process formed a stable emulsion of soluble antigen in AF. As a control, 30 μg ovalbumin were mixed with alum (Pierce Chemical Co.) at 1:1 on a vol/vol basis according to the instructions by the manufacturers, or with HBSS (1:1), and used for the injection of mice.

***In Vitro* Stimulation of CTL.** To stimulate ovalbumin-specific CTL responses, splenocytes (30×10^6) from normal or immunized mice were primed at least 14 days earlier by incubation with 1.5×10^6 irradiated EG7-ovalbumin cells in complete medium in 24-well plates at 37°C in 7% CO_2/air. Complete medium is Iscove's modified Dulbecco's medium supplemented with 10% FCS, 2 mM glutamine, gentamicin sulfate, and 2×10^{-5} M 2-mercaptoethanol.

Cytotoxic Assay. The cytotoxic activity of *in vitro*-stimulated splenocytes was determined using the ^{51}Cr-release assay (14). Briefly, EG7 target cells

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[3] The abbreviations used are: AF, antigen formulation; APC, antigen-presenting cell.

(1×10^6) were labeled with 100 μCi (1 Ci = 37 GBq) of ^{51}Cr for 60 min and washed three times with HBSS. Labeled EG7 target cells (1×10^4/well) were combined with *in vitro*-primed CTL effector cells at various effector:target ratios and incubated in 200 μl of RPMI containing 10% FCS, 2 mM glutamine, gentamicin sulfate, and 2×10^{-5} M 2-mercaptoethanol. After 4 h incubation at 37°C, 100 μl of supernatant was collected, and the amount of ^{51}Cr released from the labeled target cells was determined, as described previously (14). Spontaneous release from tumor target in the absence of CTL was <25% of maximal incorporation, as determined by detergent lysis. The percentage of cytotoxicity obtained at each effector:target ratio was calculated as:

$$\% \text{ cytotoxicity} = \frac{\text{Test release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

Tumor Growth *In Vivo*. Primary EG7 and EL-4 tumors were propagated in C57BL/6 mice by s.c. inoculation with 2×10^6 *in vitro*-propagated cells in 0.2 ml of HBSS. Tumor growth was monitored every 3–4 days by measuring length (L) and width (W) in mm, and tumor volumes (V) were determined by $L \times W^2 \div 2$. To evaluate antitumor activity of the various treatment strategies, the tumor volume of mice (6–8/group) immunized as described above were compared to a control group injected with tumor cells. Tumor measurements were determined until 50% of all animals in a treatment group had tumor volumes that exceeded 4000 mm^3. Data are presented as median tumor size. Because the EG7 tumors were propagated directly from EG7 cells grown in tissue culture, *i.e.*, no passage in mice was done, a considerable variation in tumor volume was observed among individual mice. Therefore, rank-transformed data were analyzed to determine if there were statistically significant differences among different treatment groups.

Depletion of CD4$^+$ and CD8$^+$ Cells *in Vivo*. One ml of one-tenth-diluted ascitic fluids of mAb RL.172 or mAb 3.168 in HBSS was injected i.p. into mice to deplete CD4 and CD8 cells, respectively. FACS analysis revealed that the above treatment with antibodies eliminated 95% of CD4$^+$ or CD8$^+$ cells in the spleen.

Statistical Analysis. Tumor data were analyzed for statistical significance by rank transformation, as described (16). The rank score was analyzed with Wilcoxon test. The treatment groups were compared with 0.05 experimentwise error rate (P = 0.05). The level of significance was defined as any values between 0.05 and or less.

Results

Induction of CTL by Ovalbumin in AF. Improvements in defined chemical adjuvant preparations, when mixed with soluble proteins, have been made to increase the generation of CD8$^+$ CTL (14). Initially, we tested the potency of a Squalane, Tween, Pluronic acid combination (AF) to induce antigen-specific CTL after immunization with soluble ovalbumin in AF. Mice were immunized with a single injection of 30 μg ovalbumin in saline or AF. Approximately 2 weeks later, splenocytes from immunized mice were stimulated *in vitro* with ovalbumin-expressing EG7 cells for 5 days, and cytotoxicity was determined using ^{51}Cr-labeled EG7 target cells. The results in Fig. 1 demonstrate that mice immunized with ovalbumin in AF had high levels of ovalbumin-specific CTL activity, as determined by killing of EG7 tumor target cells. No lytic activity was observed with ovalbumin-negative parent EL4 cells. Furthermore, effector cells from mice immunized with ovalbumin in HBSS demonstrated only background levels of cytolytic activity and indicated the inability of soluble proteins to induce CTL responses unless delivered in an appropriate vehicle, such as AF (14). The CTL induced in this system were demonstrated to be CD8$^+$ and MHC class I restricted (14).

Effect of Ovalbumin-AF Vaccination on Tumor Regression. To determine the *in vivo* significance of the ovalbumin/AF-induced CTL responses, different schedules of ovalbumin-AF injections were assessed as therapeutic vaccinations in EG7 tumor-bearing C57BL/6. The average tumor volume doubling time of the EG7 tumor was estimated to be 2 days. Five groups of mice were injected with EG7 cells (2×10^6), followed by immunization with 30 μg ovalbumin in AF or alum on different days after EG7 challenge. Mice immunized with ovalbumin-AF on 2 days after challenge with EG7 cells demonstrated significant inhibition of tumor growth with at least 50% of the immunized animals showing no tumor growth 27 days after vaccination (P = 0.05; Fig. 2). In contrast, all mice that received a single injection of 30 μg ovalbumin in alum had actively growing tumors 10 days after tumor cell challenge. In mice immunized with ovalbumin in AF, a significant inhibition of tumor growth over alum-treated groups could be detected as early as day 13 and persisted until the end of the study (day 27). Furthermore, mice vaccinated with ovalbumin-AF on day 9 after tumor challenge showed some tumor delay over alum-treated groups (Fig. 2), with three-eighths of treated animals showing complete tumor regression (data not shown).

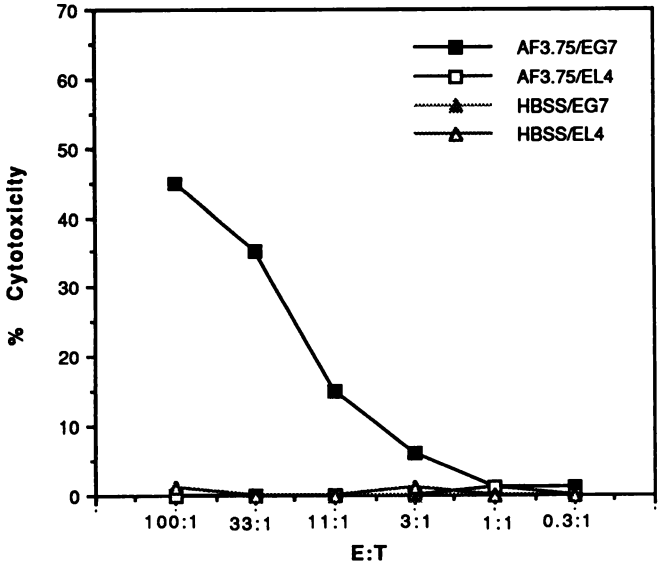

Fig. 1. Induction of CTL by ovalbumin-AF$_{3.75}$. C57BL/6 mice were immunized once with 30 μg ovalbumin in HBSS or AF$_{3.75}$. Three weeks after immunization, splenocytes were cocultured with irradiated EG7 cells at a ratio of 20:1 in complete medium in 24 tissue culture plates. The resulting effector cells were tested for CTL activity in a 4-h ^{51}Cr-release assay against EG7 or EL4 cells at the indicated effector to target ratios (E:T).

CD8$^+$ T Cells Are Involved in Tumor Regression. Since ovalbumin-AF inhibited the growth of EG7 tumors in mice, experiments were performed to analyze the phenotype of T cells responsible for the observed inhibition of tumor growth *in vivo*. This was done using a therapeutic EG7 tumor model, as described above. Initially, four groups of mice were implanted with EG7 cells. Three days later, three groups of mice were injected with ovalbumin-AF. Simultaneously, two of these groups were injected with a single i.p.-depleting dose of either anti-CD8 or anti-CD4 antibodies. As expected, ovalbumin-AF immune animals that were not depleted of CD4$^+$ or CD8$^+$ cells showed significant inhibition of tumor growth (Fig. 3), with 70% of the animals having complete tumor regression (Table 1). In contrast, mice depleted of CD8$^+$ cells completely failed to inhibit tumor growth, whereas the CD4-depleted mice showed only partial protection. These results confirm that CD8$^+$ CTL detected after vaccination with ovalbumin in AF are the primary effector cells for tumor protection. The partial protection in the CD4-depleted group may suggest that CD4$^+$ cells are necessary for priming of ovalbumin-specific CD8$^+$ cells, since the depletion of CD4$^+$ cells was done at the time of immunization and not during the effector phase of the response.

Discussion

Recent advances in the understanding of antigen presentation by APCs for T-cell recognition and activation (4, 9) have provided

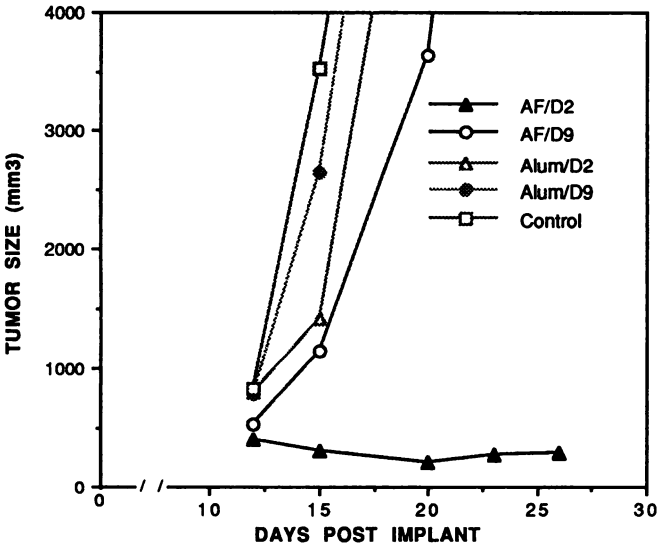

Fig. 2. The effect of ovalbumin-AF immunization on EG7 tumor regression. C57BL/6 mice, eight in each group, received s.c. injections of 2×10^6 EG7 cells. The mice were injected with 30 μg ovalbumin in AF on day 2 or 9, or with 30 μg ovalbumin in alum on day 2 or 9, after tumor challenge. The tumor size was measured at indicated time points, and the median tumor size was graphed. The normal growth kinetics of the EG7 cells was determined by setting up a control group that was injected with EG7 alone but not immunized with the antigen. Tumor volume of 4000 mm^3 was used as the target size.

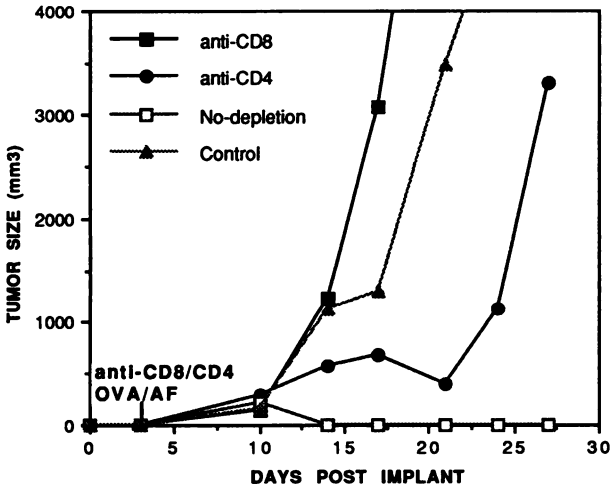

Fig. 3. Effect of $CD8^+$ T-cell depletion in tumor regression. Four groups of C57BL/6 mice received s.c. injections of 2×10^6 EG7 cells. Three groups of mice were immunized with 30 μg ovalbumin in AF on day 3. In two groups of these mice, the effect of CD8 or CD4 cell depletion on tumor regression was determined by injecting i.p. with anti-CD8 (3.168) or anti-CD4 (R172) antibodies. The other ovalbumin-AF injected group served as positive control. The normal growth kinetics of the EG7 cells was determined from the group that was injected with EG7 alone. The tumor size was measured at indicated time points, and the median tumor size was graphed.

opportunities to explore newer approaches to manipulate immune responses against cancer and infectious diseases (10–13). The present study supports and expands previous data (14), demonstrating that soluble antigen immunization in AF can generate class I-restricted T-cell responses and that such immunization protocols can be used to vaccinate animals having progressively growing tumors. Class I-restricted CTL induced by endocytic processing of tumor antigens presented via the MHC-1 molecules on the neoplastic cells play a major role in the immunity against cancer (1–3). Previously, using several different antigen systems, we demonstrated that a chemically defined antigen formulation can be used with soluble antigens to induce antigen-specific class I-restricted $CD8^+$ CTLs (14). In this study, we have extended the observation to include *in vivo* data supporting the ability of the AF to elicit tumor-specific protection in a mouse tumor model.

In an ovalbumin-expressing tumor mouse model, effector T cells from C57BL/6 mice immunized with ovalbumin-AF exhibited cytolytic response only against ovalbumin-expressing tumor cells, EG7. No lytic activity was observed against the parental tumor cells, EL4, indicating that the observed lytic response is ovalbumin specific. This lytic activity was found to be predominantly mediated by $CD8^+$ T cells and to be effective only when ovalbumin was presented on APC with $H\text{-}2^b$, but not with the $H\text{-}2^d$ molecule, indicating its genetic MHC restriction (14). The CTL priming induced by antigen-AF resembled the activity induced by transfectants and by splenocytes cytoplasmically loaded with soluble ovalbumin and recombinant vaccinia-containing antigens (8, 17). The CTLs could be restimulated in *in vitro* cultures up to 6–8 months after they were primed *in vivo* and, in terms of the functionality, the components of AF maintained their ability to induce a CTL response for at least 12 months after manufacturing (data not shown).

In a therapeutic *in vivo* tumor model, vaccination with ovalbumin-AF on days 2–3 after EG7 challenge, *i.e.*, when tumors were palpable (<50 mm^3), gave a significant tumor regression (Figs. 2 and 3). In the same model, immunization with ovalbumin-alum did not result in a significant tumor regression, although some delay in tumor growth was observed when compared to untreated controls. This suggests that the antitumor effect observed is a consequence of the immunization of ovalbumin mixed in AF. Since the EG7 tumor is a rapidly growing tumor with a doubling time of 2 days, ovalbumin-AF immunization at later time points after tumor inoculation (day 9) resulted in a lower percentage of tumor inhibition when the tumor burden at the time of vaccination was considerably larger (150 mm^3; Fig. 2). This was primarily due to the narrow window of opportunity available for the immune system to respond to the immunization and to generate effector cells that can control the rapidly growing tumor cells.

The observation that the depletion of $CD8^+$ T cells completely abolished the antigen-specific antitumor effect generated by ovalbumin-AF immunization indicates that $CD8^+$ T cells are the major effector cells involved in the antitumor protective response (Fig. 3). The finding that depletion of $CD4^+$ T cells could partially impair the ovalbumin/AF-induced antitumor effect can be explained by the fact that $CD4^+$ T cells are necessary for the priming of antigen-specific precursor $CD8^+$ T cells. The $CD4^+$ T cells could not directly kill the EG7 tumors, since EG7 cells do not express class II molecules required for $CD4^+$ T cell recognition. However, an indirect effect via cytokine production and macrophage activation cannot be ruled out. Although antibody response to ovalbumin was induced, ovalbumin synthesized in EG7 cells was genetically engineered as a secretory protein (9), and in antibody-binding and complement-mediated cytolysis assays, sera from ovalbumin-immune mice did not recognize

Table 1 *Effect of $CD8^+$ or $CD4^+$ cell depletion on tumor growth in vaccinated mice*

Treatment groups	Schedule[a] (days)	Tumor take (no. of mice)	Regression (no. of mice)
Group A		7/8	0/7
Group B	Ovalbumin/AF (No depletion)	7/7	5/7
Group C	Ovalbumin/AF + Anti-CD8 Ab	7/7	0/7
Group D	Ovalbumin/AF + Anti-CD4 Ab	5/6	2/5

[a] Three days after EG7 tumor cell challenge, 30 μg ovalbumin in AF/mouse were injected s.c. Anti-CD8 or anti-CD4 antibodies in the form of one-tenth-diluted ascites fluid were injected i.p. just before ovalbumin-AF injection. Ab, antibody.

EG7-ovalbumin cells (13). Hence, it is unlikely that the humoral response induced by ovalbumin-AF is playing a role in these tumor protection studies.

The mechanism by which the AF can drive an exogenous antigen into a processing pathway required for class I-restricted CTL response remains to be elucidated. It has been suggested that the incorporation of soluble antigens to AF may result in complexes that fuse with the cell membrane and deliver the antigens into the cytosol and to the class I-processing pathway (18). In addition, soluble antigens in AF may be taken up effectively by APCs *in vivo*, and the resulting peptides may enter into the class I pathway (19, 20). At this time, the nature of the APC involved in exogenous soluble antigen-mediated class I-restricted CTL priming is not known. Recently, the existence of specialized APCs that express class II molecules and simultaneously present antigen in class I- and class II-restricted pathways has been demonstrated (20). It is conceivable that, in our system, soluble antigen in AF could effectively be taken up by specialized APCs (19, 20, 21) and the antigen presented in the context of both class I and II molecules, as evidenced by the induction of both CTL and humoral responses. A recent study by Harding and Rui Song (21) has demonstrated that soluble antigen coupled to Latex particles can be efficiently processed by macrophages via an alternate pathway and presented in the context of class I molecules to elicit $CD8^+$ CTLs, suggesting that exogenous soluble antigens presented in a particulate form could induce antigen-specific CTLs. It is possible that soluble antigen in AF could be processed by APCs by mechanisms similar to that described by Harding and Rui Song (21). Alternatively, stimulation of CTLs may be a result of preferential activation of T_H1 *versus* T_H2 cells, which will enhance cell-mediated immune responses (22). In conclusion, we have demonstrated that immunization with soluble antigen in AF is an effective means for the induction of $CD8^+$ CTL response and tumor regression in a mouse tumor model. This approach may offer a safe and effective cancer vaccine using subcellular or recombinant tumor antigens.

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