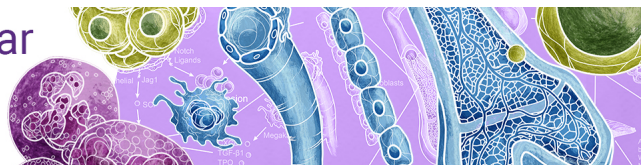


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*J Immunol* 1998; 160:3637-3641; ;  
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The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



## Cutting Edge: Liposomal Formulation of a Self Lymphoma Antigen Induces Potent Protective Antitumor Immunity

Larry W. Kwak,<sup>1\*</sup> Robin Pennington,\* Larry Boni,<sup>†</sup> Augusto C. Ochoa,<sup>2‡</sup> Richard J. Robb,<sup>†</sup> and Mircea C. Popescu<sup>†</sup>

**We developed a liposome carrier for a model nonimmunogenic, self Ag. This carrier reproducibly converted lymphoma Ig into a potent tumor rejection Ag in mice. A single immunization induced protection against challenges representing 20 to 100 times the minimum lethal dose of parental tumor. This protective effect required minimal amounts of incorporated Ag and IL-2 and elicited specific Abs (compared with free Ag or liposomal control Ig which did not elicit any specific Abs); depletion experiments demonstrated a requirement for effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Head-to-head comparisons, indicating superior potency and induction of specific T cell activation, distinguished liposomal from prototype, carrier-conjugated Ag. These results provide a strategy for formulating weak tumor or other clinically important Ags into vaccines. *The Journal of Immunology*, 1998, 160: 3637–3641.**

The ability to induce a potent, specific, and systemic immune response against inherently poor immunogenic Ags is critical to vaccine development. Various experimental strategies have been explored with candidate tumor Ags to overcome this limitation, including formulation of Ag with immunologic adjuvants, genetic fusion with immunogenic carriers, and paracrine delivery of cytokines or costimulatory molecules. However, these methods generally function to enhance the immunogenicity of Ags or tumors that are already somewhat immunogenic; only rarely do they convert a truly nonimmunogenic Ag to an immunogenic one (1–3).

Our laboratory has studied syngeneic, lymphoma-derived Ig as a model tumor Ag. The recognition sites of the B cell Ag receptor

contain determinants, which are defined by their unique variable region sequences, that can themselves be recognized as Ags. Since B cell malignancies are clonally restricted to the synthesis of a single Ig, these determinants, Ids, can serve as tumor-specific Ags (4). Lynch et al. initially demonstrated that tumor Id-specific Abs and resistance to myeloma growth could be elicited in syngeneic mice, and this observation was later supported by others (5–10). Subsequently, our studies of carrier-conjugated lymphoma-derived Id immunization in human patients showed that humoral responses could be induced against the specific Id on each patient's tumor (11).

Unmodified Ig (free Id protein) isolated from murine 38C13 lymphoma is completely nonimmunogenic in syngeneic mice. Physical modification of this lymphoma-derived Id by either chemical or genetic fusion to an immunogenic carrier protein, such as keyhole limpet hemocyanin (KLH)<sup>3</sup>, elicits Id-specific Abs; however, there is little evidence for an effector T cell response (10, 12). Liposomes can be effective vaccine carriers, inducing, in some cases, specific T cell immunity (13). Therefore, we explored a novel liposome formulation, consisting of free 38C13 Id, lipid, and human rIL-2 (liposomal lymphoma-derived Id (lipo-Id)).

### Materials and Methods

#### *Mice and tumor*

We obtained 6 to 12-wk-old C3H/HeN Mtv-negative female mice from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research and Development Center (NCI-FCRDC), Frederick, MD. The carcinogen-induced, C3H 38C13 B cell lymphoma has been previously described (14).

#### *Liposomal vaccine preparation*

Free IgM (38C13 Id) was isolated by somatic cell hybridization (15), and 38C13 Id and a control IgM<sub>kappa</sub> (4C5) (both from R. Levy, Stanford University, Stanford, CA) were purified from ascites. Where indicated, a second control IgM<sub>kappa</sub> (TEPC-183, Sigma, St. Louis, MO) was used. Id-KLH was prepared by glutaraldehyde conjugation at a 1:1 ratio of Id and KLH (10). The liposomal entrapment procedure of Anderson (16) was modified as follows: Aqueous Id, IL-2, and mouse serum albumin were added to dry, powdered dimyristoylphosphatidylcholine, warmed to 37°C, and subjected to three cycles of vortexing, sonication, freezing at –70°C, and thawing at 37°C. Following dilution with 1 mM PBS (pH 7), samples were washed three times by centrifugation (12,000 revolutions per min). Final concentrations were typically 40 mM dimyristoylphosphatidylcholine, >5 × 10<sup>5</sup> international units/ml IL-2, and various amounts of Id Ag

\*Department of Experimental Transplantation and Immunology, Medicine Branch, Division of Clinical Sciences, National Cancer Institute, Bethesda, MD 20892; <sup>†</sup>Biomira USA, Cranbury, NJ 08512; and <sup>‡</sup>SAIC-Frederick, Frederick, MD 21702

Received for publication December 2, 1997. Accepted for publication February 11, 1998.

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<sup>1</sup> Address correspondence and reprint requests to Dr. Larry W. Kwak, National Cancer Institute, Frederick Cancer Research and Development Center, Bldg. 567, Rm. 205, Frederick, MD 21702. E-mail address: kwak@ncifcrf.gov

<sup>2</sup> Current address: Louisiana State University Medical Center, New Orleans, LA 70112.

<sup>3</sup> Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; lipo-Id, liposomal lymphoma-derived Id.

as indicated. Liposomes had a bimodal distribution consisting of both small (mean diameter of 177 nm) and medium/large (mean diameter of 2  $\mu\text{m}$ ) vesicles. Based on analysis with an anti-38C13 Id mAb (S1C5, M. Kaminski, University of Michigan, Ann Arbor, MI) and electron microscopy, Id and IL-2 were distributed on both the outer surface of the vesicles and between the inner lipid bilayers.

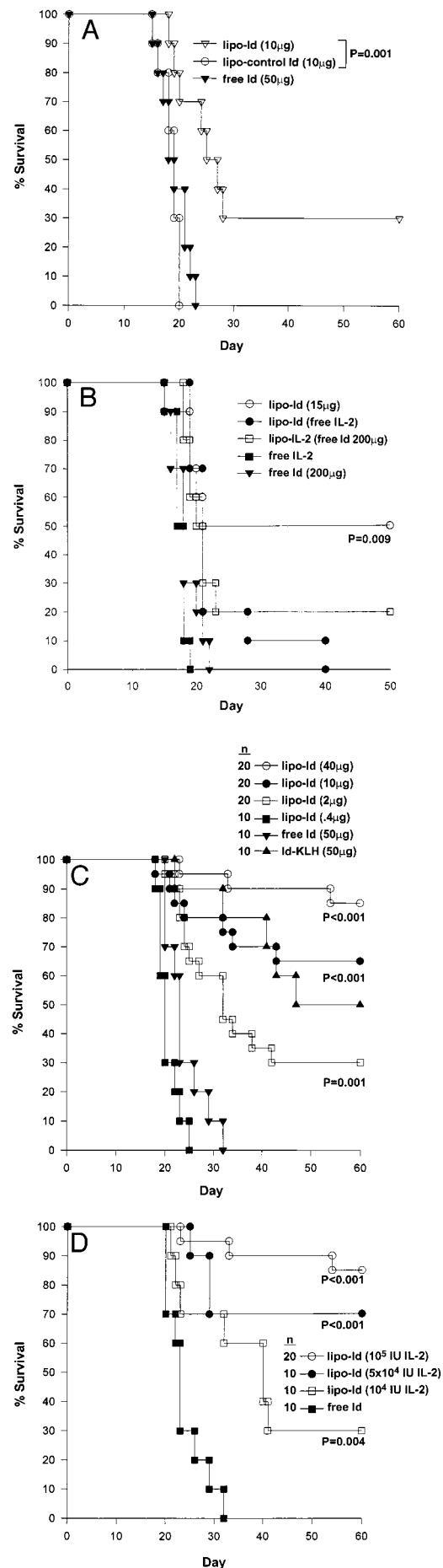
The concentration of incorporated Id for each individual preparation was determined by sandwich ELISA after dissolution of lipid vesicles with detergent. The unknown was captured by rabbit anti-mouse IgM and detected by biotinylated rabbit anti-mouse IgM (17). Streptavidin-europium was added as a final step, and europium fluorescence was recorded by a 1232 delayed fluorescence immunoassay time-resolved fluorometer (Wallac, Gaithersburg, MD). Experiments with a control IgM demonstrated that similar exposure to detergent did not influence detection by ELISA. Furthermore, the sum total of incorporated and unincorporated Id was equivalent to the amount of input Id, based on protein content, within a few percentage points (data not shown).

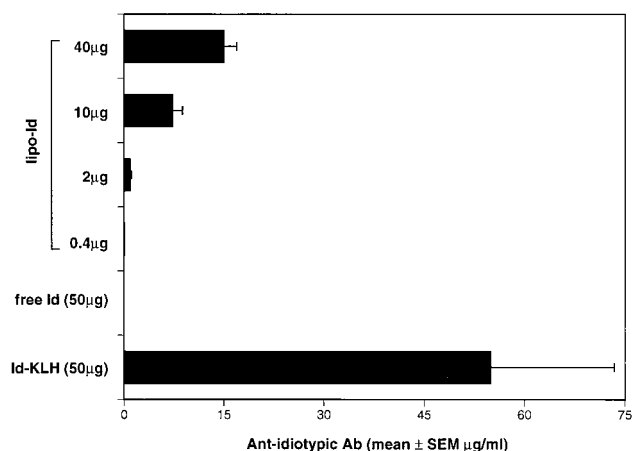
## Results and Discussion

Mice (10 per group) that had been previously immunized with lipo-Id and challenged with 20 times the minimum lethal dose of lymphoma 2 wk later demonstrated significantly prolonged survival as well as protective immunity (30%, Fig. 1A). This was in distinct contrast to controls that had been immunized either with liposomes containing comparable amounts of a control IgM (lipo-control Id,  $p = 0.001$ ) or with free Id ( $p = 0.002$ ). The potency of lipo-Id was also apparent against a substantially larger tumor inoculum (100 times the minimum lethal dose;  $p = 0.001$  vs control liposomes, data not shown). The requirement for liposomal incorporation of Id or IL-2 was evaluated by the experiment shown in Figure 1B. Formulations consisting of both Id and IL-2 entrapped in liposomes (lipo-Id), Id only in liposomes that was then mixed with an equivalent amount of free IL-2 (lipo-Id (free IL-2)), or IL-2 only in liposomes subsequently mixed with free Id (lipo-IL-2 (free Id)) were used to immunize mice (10 per group), followed by tumor challenge. Optimal protective immunity was observed in mice immunized with liposomes containing both Id and IL-2 ( $p < 0.01$  compared with controls immunized with either free Id or IL-2 alone), suggesting that physical entrapment of Ag and IL-2 was critical.

Given the above results, we sought to optimize Ag incorporation. Four lipo-Id vaccines, delivering Id doses of 0.4 to 40  $\mu\text{g}$ /mouse, were prepared by serially diluting the amount of input Id while holding the concentrations of other components constant. Mice given a single immunization with lipo-Id and challenged with tumor demonstrated a clear dose-dependent effect on protec-

**FIGURE 1.** Immunization with liposomal, but not free, tumor-derived Id elicits protective antitumor immunity. *A*, Id or a control IgM (lipo-control Id) was incorporated into liposomes; the dose of Id administered per mouse is indicated in parentheses. Mice (10 per group) were immunized i.p. and challenged i.p. with  $2 \times 10^3$  lymphoma cells 2 wk later. *B* indicates the requirement for both Ag and IL-2 incorporation. Formulations consisted of both Id and IL-2 entrapped in liposomes (lipo-Id), Id only in liposomes that was then mixed with an equivalent amount of free IL-2 (lipo-Id (free IL-2)), or IL-2 only in liposomes subsequently mixed with free Id (lipo-IL-2 (free Id)). *C* shows the effect of Ag dose and a comparison of potency with KLH-conjugated Id. Serial dilutions of input Id were used to prepare liposomes containing equal concentrations of other components. *D* shows IL-2 dose response. Serial dilutions of input IL-2 were used to prepare liposomes containing equal concentrations of other components (40  $\mu\text{g}$  Id per mouse). *B–D*, Mice (10–20 per group) received a single i.p. immunization and were then challenged with tumor as in *A*. Statistical comparisons of survival (as compared with free Id) were made on the basis of nonparametric Mantel-Cox log rank  $p$  values (BMDP Statistical Software, Los Angeles, CA).



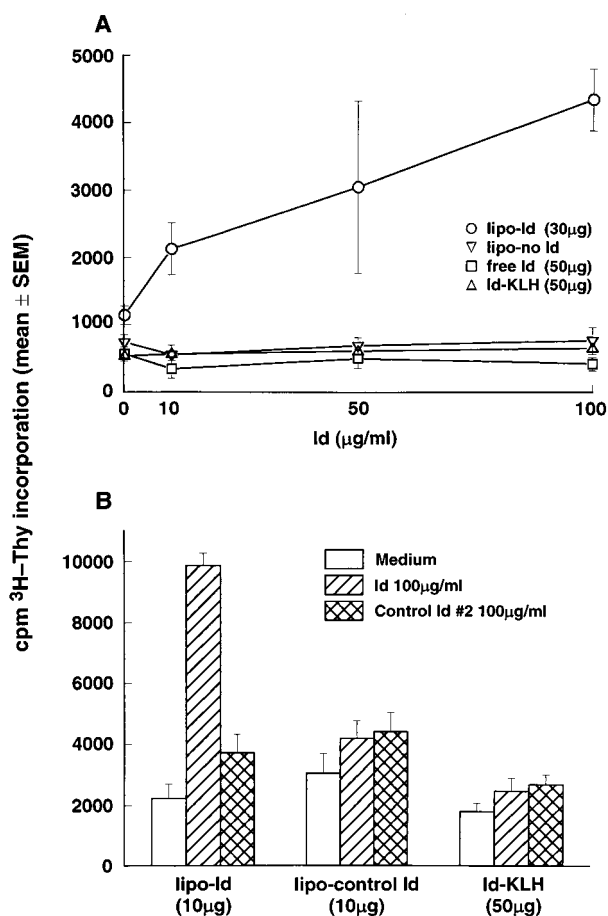


**FIGURE 2.** Humoral responses. Mean antiidiotypic Ab levels of five mice/group from the experiment in Figure 1C were determined at the time of tumor challenge. Serum was serially diluted over microtiter plates coated with lymphoma-derived Id which had been affinity purified using an anti-Id mAb. Binding of serum Abs to Id was detected by goat anti-mouse IgG horseradish peroxidase-conjugated Abs. Serum antiidiotypic Ab levels were quantitated by comparing sera titration curves with a standard curve obtained with a known concentration of a mixture of purified anti-Id mAbs. Specificity for Id was demonstrated by the lack of binding to a control IgM (TEPC-183, data not shown).

tive immunity, with those vaccines delivering 40, 10, and 2  $\mu\text{g}$  Id producing superior survival compared with free Id (Fig. 1C). In this experiment, we also compared the potency of lipo-Id with a previously determined optimal dose of KLH-conjugated Id (50  $\mu\text{g}$  Id). Matched for Ag dose, the protective effect of lipo-Id (40  $\mu\text{g}$  Id) was superior to that of Id-KLH ( $p = 0.036$ , Fig. 1C). In a separate experiment, the superior potency of lipo-Id was also apparent at suboptimal, lower Ag doses. Nine mice immunized with lipo-Id (2  $\mu\text{g}$  Id) demonstrated 33% protection, while 10 mice per group immunized with Id-KLH containing 50, 10, or 2  $\mu\text{g}$  Id per mouse demonstrated 40, 30, and 0% protection, respectively ( $p = 0.007$  for lipo-Id vs Id-KLH (2  $\mu\text{g}$  Id), data not shown). Neither free nor liposomal IL-2 (without Ag) enhances protective immunity when mixed with either free Id or Id-KLH in this model (18); thus, the superiority of lipo-Id over Id-KLH cannot be attributed to the simple addition of IL-2.

In parallel, input IL-2 was serially diluted while the dose of Id (40  $\mu\text{g}$ ) was held constant. A clear IL-2 dose-dependent effect on protective immunity was also observed (Fig. 1D). It was striking that vaccines containing as little as 1/10 the prototype amount of IL-2 were capable of inducing significant protection ( $p = 0.004$  vs free Id).

To investigate the mechanism by which lipo-Id induces protective antitumor immunity, we initially determined antiidiotypic Ab levels elicited by the vaccines in Figure 1C (Fig. 2). A clear dose-dependent effect of encapsulated Id was apparent on the Ab response (15, 7, 1, and 0.1  $\mu\text{g}/\text{ml}$ , respectively). The induction of a humoral response by lipo-Id was distinct from free Id, which failed to induce any Ab. However, compared with Id-KLH immunization (55  $\mu\text{g}/\text{ml}$ ), these levels of Ab were uniformly low, even though two lipo-Id preparations had produced superior or equivalent tumor protection, respectively (Fig. 1C). Both lipo-Id and Id-KLH predominantly induced IgG1 isotype Abs; some IgG2a isotype Abs were also induced (data not shown). The apparent lack of correlation between Ab response and tumor protection suggested

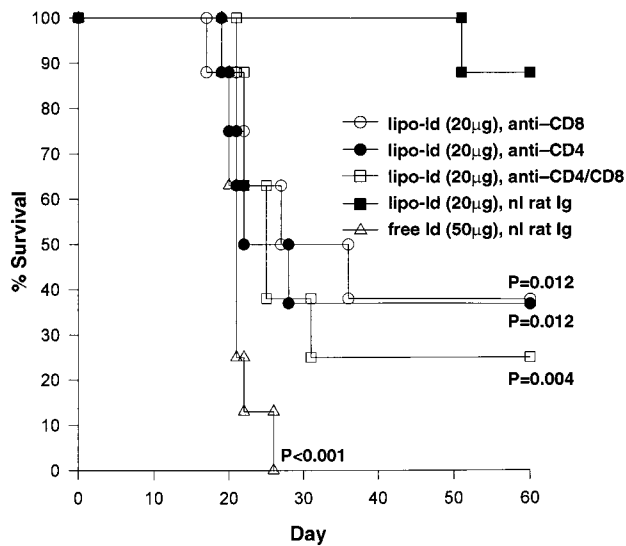


**FIGURE 3.** In vitro Id-specific T cell responses. Results from two separate experiments are shown. Splenocytes obtained from two to three mice/group that had been immunized i.p. 2 wk earlier as indicated were pooled and enriched for T cells by passing over nylon wool and then placed in 96-well flat-bottom microtiter plates in quadruplicate (200  $\mu\text{l}$ ,  $2 \times 10^5$  cells/well) with free Id at various concentrations (A), or with a single concentration of Id or one of two control IgMs (B). Normal syngeneic splenocytes (2000 rad,  $5 \times 10^5$ ) were added as a source of APCs. Cultures were maintained at 37°C, 5%  $\text{CO}_2$  for 5 to 7 days, and 18 to 24 h before harvesting 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine (2 Ci/mmol, New England Nuclear Research Products, Boston, MA) in 50  $\mu\text{l}$  medium was added to each well. Incorporated radioactivity was measured in an LKB 1205 Betaplate liquid scintillation counter (Gaithersburg, MD).

that Ab could not fully account for the protection induced by lipo-Id.

Next, we investigated splenic T cell proliferation in vitro. The representative experiment shown in Figure 3A demonstrates a dose-dependent response to free Id after priming with lipo-Id, but not after priming with liposomes without Ag, Id-KLH, or free Id. The specificity of the response for Id was formally demonstrated in a separate experiment by both the lack of parallel response to a control IgM (4C5) and the lack of response to 38C13 Id after priming with liposomes containing another control IgM (lipo-control Id, TEPC-183, Fig. 3B). Thus, at a lipo-Id concentration (10  $\mu\text{g}$ ) that produced tumor protection equivalent to that of Id-KLH, there was a profound difference in the ability of the former, but not the latter, to induce T cell activation.

To definitively determine the role of effector T cells in protection, we depleted specific subsets in vivo in immunized mice. Mice primed with lipo-Id received depleting mAbs that were specific for



**FIGURE 4.** Effect of in vivo T cell depletion. Mice received a single i.p. immunization with lipo-Id and were randomly assigned to receive treatment with a depleting mAb specific for CD4<sup>+</sup> or CD8<sup>+</sup> T cells (GK1.5 and 53.6–72, respectively, ammonium sulfate purified ascites, preclinical repository, NCI-FCRDC), both mAbs, or with normal rat IgG (Sigma) 2 wk later on every other day for three i.p. doses (0.4 mg/day, eight mice/group). Controls immunized with free Id also received treatment with normal rat IgG. All mice were then challenged i.p. with  $2 \times 10^3$  lymphoma cells. Flow cytometry (27) analysis of splenocytes from normal mice treated with these mAbs in parallel 1 and 2 wk after treatment confirmed a >95% depletion of the appropriate subset with normal levels of the other subset. *p* values refer to comparisons with non-T cell-depleted, lipo-Id-vaccinated mice.

either CD4<sup>+</sup> or CD8<sup>+</sup> T cells (or both) or normal rat IgG before tumor challenge (Fig. 4). Depletion of either subset, either alone or in combination, was associated with markedly reduced survival ( $p = 0.012$  and  $0.004$ , respectively, vs rat IgG-treated controls), suggesting a requirement for both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. However, these results do not formally exclude a minor role for anti-idiotypic Abs that are found intact in mAb-treated mice (data not shown), especially given the incomplete abrogation of protection.

These experiments demonstrate the successful conversion of nonimmunogenic, free lymphoma-derived Id into a vaccine, without structural modification of the Ag by chemical or genetic fusion. As a “self” Ag, Id provided a rigorous test for this carrier and, consequently, a relevant model for other poorly immunogenic tumor or infectious disease Ags. The potency of this formulation was apparent by both the small amount of incorporated Ag actually required, and by comparison with the optimal dose of prototype, carrier-conjugated Id. It is possible, however, that the reduction in Ag dose required for immunogenicity may vary for other tumor Ags.

The precise cellular mechanisms through which T cells induced by lipo-Id mediate antitumor immunity remain to be elucidated. Liposomes are known to target Ag to lymphoid organs for processing in either MHC class I or II presentation pathways by APCs (19, 20). However, IL-2 incorporation in the same vesicle was also required. Preliminary studies suggest that the sustained release of IL-2 occurs, and that IL-2 on the vesicle surface may be capable of causing the association and internalization of liposomes with cells expressing IL-2R (M.C. Popescu, unpublished observations). Fur-

ther studies of in situ interactions between APCs, T cells, and other potential non-T cell effectors are in progress.

The ability of lipo-Id to induce Id-specific T cells also distinguishes this formulation from Id-KLH (Fig. 3), which is currently being tested in clinical trials (11, 21), as well as from Id-cytokine fusions (22) and DNA vaccines (23, 24), none of which have been reported to prime for a CD4<sup>+</sup> or CD8<sup>+</sup> T cell response to Id in murine models. Dendritic cells pulsed with Id may represent an alternative vaccine to induce T cell immunity (25).

Finally, evidence suggesting that T cells recognize idiotypic determinants as processed peptides with MHC was provided by the unexpected cross-reactivity of murine CD8<sup>+</sup> influenza hemagglutinin-specific T cell clones with a 10 amino acid peptide that was derived from a myeloma Ig heavy chain variable region (26). Furthermore, we recovered a human donor-derived myeloma Id-specific CD4<sup>+</sup> T cell line from a marrow transplant recipient that was blocked by anti-MHC class II Abs (21).

Future studies with this potent carrier should be aimed at formulating other cytokines of potential interest for vaccine development. Ultimately, the simple and reproducible formulation of this carrier should facilitate its translation to clinical trial.

## Acknowledgments

We thank Ronald E. Gress for critically reading the manuscript and Steve Weeks for technical assistance.

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