Human Autologous Tumor-Specific T-Cell Responses Induced by Liposomal Delivery of a Lymphoma Antigen

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ABSTRACT

Purpose: The idiotype (Id) of the immunoglobulin on a given B-cell malignancy is a clonal marker that can serve as a tumor-specific antigen. We developed a novel vaccine formulation by incorporating Id protein with liposomal lymphokine that was more potent than a prototype, carrier-conjugated Id protein vaccine in preclinical studies. In the present study, we evaluated the safety and immunogenicity of this vaccine in follicular lymphoma patients.

Experimental Design: Ten patients with advanced-stage follicular lymphoma were treated with five doses of this second generation vaccine after chemotherapy-induced clinical remission. All patients were evaluated for cellular and humoral immune responses.

Results: Autologous tumor and Id-specific type I cytokine responses were induced by vaccination in 10 and 9 patients, respectively. Antitumor immune responses were mediated by both CD4+ and CD8+ T cells, were human lymphocyte antigen class I and II associated, and persisted 18 months beyond the completion of vaccination. Specific anti-Id antibody responses were detected in four patients. After a median follow-up of 50 months, 6 of the 10 patients remain in continuous first complete remission.

Conclusions: This first clinical report of a liposomal cancer vaccine demonstrates that liposomal delivery is safe, induces sustained tumor-specific CD4+ and CD8+ T-cell responses in lymphoma patients, and may serve as a model for vaccine development against other human cancers and infectious pathogens.

INTRODUCTION

Immunoglobulin molecules expressed on the surface of B cells act as B-cell antigen receptors. The variable regions of the heavy and light chains of the immunoglobulin molecules combine to form the antigen-binding sites. Each immunoglobulin molecule has a unique variable region sequence, termed idiotype (Id), that can also be recognized as an antigen. Malignancies of mature and resting B cells arise from clonal proliferation of cells that express immunoglobulins with unique variable region sequences on their surface. The Id on a given B-cell malignancy can therefore serve as a tumor-specific antigen and can be exploited as a target for active specific immunotherapy (1–3).

Several preclinical studies have shown that immunization of animals with tumor-specific Id protein can induce resistance to growth of syngeneic tumors (1–8). Based on these preclinical observations, individualized Id vaccines have been evaluated in a number of clinical trials in lymphoma and myeloma patients (9–16). Principal among these studies was the demonstration that autologous Id protein can be formulated into an immunogenic antigen in follicular lymphoma (FL) patients, when conjugated to a carrier protein, keyhole-limpet hemocyanin (KLH), and administered together with granulocyte macrophage colony-stimulating factor (GM-CSF) as an adjuvant (11). Vaccination of patients in complete clinical remission with Id-KLH + GM-CSF was associated with induction of complete molecular remissions and long-term disease-free survival (11). However, the chemical conjugation of Id to KLH produces a heterogeneous final product, which makes it difficult to characterize and control for quality. Furthermore, vaccine formulations of increased potency are probably needed to achieve meaningful clinical benefit in patients with partial remission or bulky disease.

Liposomes have been shown to be effective carriers for peptide and protein antigens in animal studies (17, 18). We developed a novel vaccine formulation by incorporating the Id into a uniform liposomal carrier, containing dimyristoylphosphatidylcholine lipid. Recombinant human interleukin (IL)-2 was also incorporated into the liposomes as an adjuvant. This formulation (liposomal Id/IL-2) produced a more homogeneous final product and reproducibly converted the lymphoma Id into a tumor rejection antigen in a mouse lymphoma model (19). Furthermore, in head to head comparisons, this liposomal vaccine was found to be more potent than the Id-KLH vaccine. Depletion experiments suggested that both CD4+ and CD8+ T cells were required for protection (19). Thus, this formulation induced both helper and cytotoxic T-cell immune responses, an essential feature for optimal antitumor immunity.

We have now evaluated the safety and immunogenicity of
this novel vaccine formulation in FL patients. Ten patients with advanced-stage FL, previously induced into clinical remission with a uniform chemotherapy regimen, were given five doses of the liposomal Id/IL-2 vaccine subcutaneously at approximately monthly intervals. Patients had minimal adverse effects at the sites of injections, and no grade 3 or grade 4 adverse events related to the vaccine were noted. Immunologic studies revealed that all 10 patients developed antitumor T-cell responses, and 4 patients developed anti-Id antibody responses as well. After a median follow-up of 50 months, 6 of 10 patients remain in continuous first complete remission.

PATIENTS AND METHODS

Patients. After obtaining signed informed consent, 10 previously untreated patients with stage II–IV FL grade 1 or 2 were enrolled on this investigational review board-approved phase I clinical trial. All patients underwent a lymph node biopsy before starting treatment to obtain tissue for vaccine production. The lymph node specimen was processed into a single cell suspension and cryopreserved. Patients were treated uniformly with the chemotherapy regimen PACE (ProMACE without methotrexate; prednisone, doxorubicin, cyclophosphamide, and etoposide). Chemotherapy was administered on days 1 and 8 of a 28-day cycle (20). Patients received a minimum of six cycles of chemotherapy or until two cycles past the best clinical response to achieve minimal residual disease state. Clinical responses were assessed by physical examination, computerized tomography scans, lymphangiograms, and bilateral bone marrow biopsies. Nine patients achieved complete clinical response and one achieved partial response to the chemotherapy bone marrow biopsies. Nine patients achieved complete clinical response to achieve minimal residual disease state.

1 and 8 of a 28-day cycle (20). Patients received a minimum of 106 IU of IL-2, and 160 mg of dimyristoylphosphatidylcholine that was used to generate liposomes. Before administration, the contents of the vial were diluted in 2 mL of normal saline and injected subcutaneously at four separate sites in arms and legs at a dose of 0.5 mL per site. Approximately 6 months after the completion of the chemotherapy, to allow time for immunologic recovery, all patients were given five doses of the vaccine at months 0, 1, 2, 3, and 5, while they were still in clinical remission.

Peripheral Blood Mononuclear Cells. Blood samples were obtained from patients at various time points before and after vaccination, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation with Ficoll Isopaque (ICN Biomedicals Inc., Aurora, OH) and cryopreserved for immunologic assays. Pre- and postvaccine PBMCs were thawed, washed, and resuspended to a concentration of 1 to 3 × 106 cells per mL in RPMI 1640 with 1× Glutamax (Invitrogen, Carlsbad, CA), supplemented with 5% fetal bovine serum (Hyclone, Logan, UT), 1 mmol/L sodium pyruvate (Bio-Whittaker, Walkersville, MD), 20 mmol/L HEPES buffer (Invitrogen), 50 μmol/L β-mercaptoethanol (Sigma, St. Louis, MO), 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen), and 10 μg/mL gentamicin (BioWhittaker) [complete medium]. Five milliliters per well of the suspensions were plated into 6-well plates (Corning, Inc., Corning, NY) and rested overnight at 37°C in 5% CO2 in air. The next day, PBMCs were harvested and washed before use in immunologic assays.

Activation of Tumor Cells and Normal B Cells. Cryopreserved cells from the lymph node biopsy specimen were enriched for tumor cells by depletion of T cells with CD3 microbeads over a magnetic column (Miltenyi Biotec, Auburn, CA) using the manufacturer’s protocol. Autologous normal B cells were isolated from PBMCs by magnetic cell separation method using the B Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer’s protocol. The purity of the isolated tumor and normal B cells was >95%. Tumor cells and normal B cells were activated for 3 days with recombinant human soluble CD40 ligand trimer (800 ng/mL; sCD40L; Amgen, Thousand Oaks, CA) and recombinant human IL-4 (2 ng/mL; Peprotech, Rocky Hill, NJ). Activated tumor cells and normal B cells were harvested and washed before coculture with PBMCs.

Table 1 Patient characteristics

<table>
<thead>
<tr>
<th>UPN</th>
<th>Age (y)/sex</th>
<th>Clinical stage</th>
<th>Histology/grade</th>
<th>Response to chemotherapy</th>
<th>Off therapy (mo) *</th>
<th>Status (mo) †</th>
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<tbody>
<tr>
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<td>FL/1</td>
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<td>CCR (60+)</td>
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<td>FL/2</td>
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<td>CR</td>
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Abbreviations: UPN, unique patient number; CR, complete response, PR, partial response; PD, progressive disease; CCR, continuous first complete response.

* Months between the end of chemotherapy and vaccine treatment.
† Months after completion of chemotherapy.
‡ Developed progressive disease at 11 months after chemotherapy and then went into spontaneous remission at 28 months.
§ Developed secondary acute myeloid leukemia 25 months after the completion of chemotherapy and eventually died.
Our pilot experiments indicated that sCD40Lt-activated tumor cells markedly enhanced the sensitivity of our immunologic assays by increasing the cytokine production by responding T cells as compared with unmodified tumor cells (data not shown). We have therefore used sCD40Lt-activated tumor cells as stimulators to evaluate T-cell responses in these patients.

**Cytokine Induction Assay.** Pre- and postvaccine PBMCs (1 × 10^6/mL) were cultured in complete medium in a 48-well plate in the absence or presence of sCD40Lt-activated autologous tumor cells (0.5 × 10^6 cells per mL) or autologous normal B cells (0.5 × 10^6 cells per mL; ref. 11). Supernatants were harvested after 2 days and 6 days of incubation at 37°C in 5% CO_2 in air, and cytokine production [interferon (IFN) γ, GM-CSF, and tumor necrosis factor (TNF)-α] was measured by enzyme-linked immunosorbent assay (ELISA) using Quantikine kits (R&D Systems, Minneapolis, MN). TNF-α and GM-CSF were assayed in the supernatants after 2 days of incubation, and IFNγ was assayed in the supernatants after 6 days of incubation. These time points were found to be optimal for the detection of the respective cytokines in our pilot experiments (data not shown). A positive response was defined as a response ≥ 2× that of the negative controls, which included postvaccine PBMCs. In selected patients, CD4^+ and CD8^+ T cells were isolated using antihuman lymphocyte antigen (HLA) class I and class II blocking antibodies (BD Pharmingen, San Diego, CA) before coculturing with PBMCs. In selected patients, CD4^+ and CD8^+ T cells were isolated from PBMCs using CD4^+ and CD8^+ T cell isolation Kits (Miltenyi Biotec) using the manufacturer’s protocol. The purity of the isolated CD4^+ and CD8^+ T cells was >95%.

To assess Id-specific cytokine production, fresh or cryopreserved PBMCs (1 × 10^6 cells per mL) were cultured in Clicks medium supplemented with 10% fetal bovine serum (Hyclone), 2% human AB serum (Gemini, Calabasas, CA), and the same additional ingredients as mentioned above in a 48-well plate in the absence or presence of patient-specific Id protein (50 μg/mL) or control isotype-matched irrelevant Id proteins (11). Supernatants were harvested after 6 days of incubation at 37°C in 5% CO_2 in air, and cytokine production (IFNγ, GM-CSF, and TNF-α) was measured by ELISA (R&D Systems). A positive response was defined as a response ≥ 2× that of the negative controls (PBMCs alone or PBMCs + irrelevant Id).

**Interferon-γ Enzyme-Linked Immunospot Assay.** The IFNγ enzyme-linked immunospot (ELISPOT) assay was done as described previously (22). The precursor frequency of IFNγ-producing T cells was determined by subtracting the background spots in tumor alone and PBMCs alone from the number of spots seen in response to tumor cells. Significant difference in the precursor frequency of tumor-reactive T cells between the pre- and postvaccine samples was determined by using the Student’s t test for paired mean values.

**Anti-Idiotype Antibody Assay.** The anti-Id antibody responses were measured by ELISA as described previously (9). A microtiter plate was coated with patient-specific Id protein or control isotype-matched irrelevant Id proteins. Pre- and postvaccine serum samples were serially diluted over the Id- and irrelevant Id-coated wells. Bound antibody was detected with horseradish peroxidase-conjugated goat antihuman light chain or heavy chain antibodies (Caltag, Burlingame, CA) directed against the light chain or heavy chain not present in the autologous Id. A positive response was defined when the anti-Id antibody titer increased ≥4-fold.

**RESULTS**

**Autologous Tumor-Specific CD4^+ and CD8^+ T-Cell Responses Were Induced in All Patients by Immunization.** Cryopreserved pre- and postvaccine PBMCs were tested in parallel for recognition of primary autologous tumor cells after a single 2- to 6-day stimulation. Tumor cells were first isolated by magnetic cell separation and activated with sCD40Lt (see Patients and Methods). Activation of B-cell tumor cells with sCD40Lt up-regulated various costimulatory molecules and MHC class I and class II molecules on the surface of tumor cells, associated with enhanced antigen-presenting capability (data not shown; refs. 23 and 24). Postvaccine PBMCs from all 10 patients responded to sCD40Lt-activated autologous tumor cells by producing significant amounts of IFNγ, GM-CSF, and TNF-α compared with PBMCs or tumor alone (Fig. 1A–C). The PBMC response against the tumor cells was confirmed on samples from multiple postvaccine time points in each patient (data not shown). Importantly, there was no significant production of cytokines by prevaccine PBMCs when cocultured with the same autologous tumor cells.

Assessment of postvaccine T-cell response in one patient with adequate tumor cells (UPN 4) revealed production of type I cytokines (TNF-α, GM-CSF, and IFNγ) but not of type II cytokines (IL-4, IL-5, and IL-10) in response to autologous tumor cells (data not shown). These data were consistent with results from our previous studies with Id vaccines in FL patients, in which we generally observed type I but not type II tumor-specific cytokine responses.

To test specificity of the T-cell responses, postvaccine PBMCs were cocultured with either sCD40Lt-activated autologous normal B cells or activated tumor cells in parallel. Significant production of cytokines by prevaccine PBMCs when cocultured with the same autologous tumor cells.

Tumor-specific T-cell responses were further characterized using antihuman lymphocyte antigen (HLA) class I and class II blocking antibodies or by isolation of CD4^+ and CD8^+ T-cell subsets from postvaccine PBMCs. Compared with isotype control antibody treatment, in all 10 patients, cytokine induction was at least partially inhibited by either anti-HLA class I or class II blocking antibodies, suggesting

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*S. Neelapu and L. Kwak, unpublished observations.*
that both CD8⁺ and CD4⁺ T cells were involved in the antitumor immune responses. Representative data from six patients are shown in Fig. 3A–C. Consistent with this result, cytokine production was observed when either purified CD4⁺ or CD8⁺ T-cell subsets were cocultured with autologous tumor cells (Fig. 3D).

Cytotoxic activity was assessed in two patients (UPN 6 and 7) with availability of tumor cells and PBMCs. In both patients, significant lysis of unmodified, native autologous tumor cells (24% at an effector to target ratio of 50:1 in UPN 6 and 42% at an effector to target ratio of 25:1 in UPN 7) was induced by postvaccine T cells, but not prevaccine T cells (data not shown). There was no significant lysis of autologous normal B cells, suggesting that the lysis of tumor cells by postvaccine T cells was specific.

**Fig. 2** Cytokine immune responses in postvaccine PBMCs were specific to autologous tumor cells. Cryopreserved postvaccine PBMCs were cultured in medium alone or with sCD40L-activated autologous tumor cells or sCD40L-activated autologous normal B cells. Cytokine production in the supernatants was measured by ELISA. Representative data from five patients. A, IFNγ production; B, GM-CSF production; C, TNF-α production.

**Tumor-Specific T-Cell Responses Were Quantitated Using an Interferon-γ Enzyme-Linked Immunospot Assay.** We developed a modified IFNγ ELISPOT assay to quantitate the T-cell response to autologous tumor cells in selected patients (22). Triplicate wells demonstrating the IFNγ spots produced by pre- and postvaccine PBMCs analyzed in parallel from one representative patient are shown in Fig. 4A. The calculated precursor frequency of tumor-reactive T cells was significantly increased in postvaccine PBMCs (range, 36–141 IFNγ spots per 100,000 PBMCs), compared with prevaccine PBMCs (range, 1–49 IFNγ spots per 100,000 PBMCs) in all six patients who were evaluated (Fig. 4B; *P* < 0.05 using Student's *t* test). In three patients, we have further shown that the tumor-reactive T cells persisted for more than 18 months after completion of the vaccination (Fig. 4C and D).
Idiotype Antigen-Specific Cellular Responses Were Induced by Immunization. Specific cellular responses against autologous protein antigen (Id) were assessed by a cytokine induction assay. Freshly obtained postvaccine PBMCs from 9 of 10 patients responded specifically to autologous Id protein by producing significant amounts of cytokines (IFNγ, GM-CSF, and/or TNF-α) in a single 6-day cytokine induction assay, compared with no antigen or a panel of class-matched Id proteins (Irrel. Id) from other patients (Fig. 5A–C). These responses have been confirmed on at least two different postvaccine time points in each patient (data not shown). The Id responses could not be assessed in fresh prevaccine samples due to logistical reasons. However, comparison of cryopreserved pre- and postvaccine PBMCs from three patients in parallel showed that there was no significant production of cytokines by prevaccine PBMCs in response to Id (Fig. 5D–F).

Idiotype Antigen-Specific Antibody Responses Induced in Four Patients. Antibody responses against Id protein antigen were measured by ELISA (see Patients and Methods). Four (UPN 1, UPN 4, UPN 6, and UPN 9) of 10 patients had clear evidence of specific anti-Id antibody responses (Fig. 6A–D). Antibody responses were specific to each patient’s autologous Id because there was no binding to a panel of isotype-matched irrelevant Ids obtained from other patients in the same assay.

Clinical Outcome. The vaccine was well tolerated by all patients. Erythema and induration lasting up to a week were noted at the sites of injections in all patients. Local subcutaneous nodules were also noted in two patients, possibly related to the inflammation induced by IL-2 in the vaccine formulation. There were no adverse events grade 2 due to the vaccine. Seven patients remained in continuous complete remission, and three patients (UPN 3, UPN 7, and UPN 8) had evidence of progressive disease at the end of the vaccination (Table 1). Spontaneous complete clinical remission was subsequently observed in patient 7 approximately 17 months after the completion of the vaccination. Patient 10 developed secondary acute myeloid leukemia 25 months after the completion of the chemotherapy and eventually died. After a median follow-up of 50 months, 6 of 10 patients treated on this study remain in continuous first complete remission.

DISCUSSION

To our knowledge, this is the first report of a human cancer vaccine trial of a defined tumor antigen encapsulated in a
liposomal lymphokine delivery system. This trial establishes that liposomes act as efficient carriers of Id protein and IL-2 and that administration of this novel vaccine formulation to FL patients after chemotherapy-induced remission is safe and can induce sustained, tumor-specific CD4\(^+\) and CD8\(^+\) T-cell responses lasting longer than 18 months after vaccination. Several possible mechanisms likely contribute to the potency of this liposomal vaccine formulation. Liposomes probably provide a depot effect and cause a sustained release of antigen and IL-2 over several days to weeks (25, 26). In addition, liposomes have been shown to preferentially distribute via lymph and reach local lymphoid organs after subcutaneous administration (27, 28). Reports in the literature also indicate that the antigen encapsulated in liposomes is delivered into both the endosomal and cytosolic processing pathways of antigen-presenting cells, thereby generating both CD4\(^+\) and CD8\(^+\) T-cell responses (25, 29, 30).

Recognition of tumor antigens that are naturally processed and presented in the context of MHC class I and class II molecules on the surface of tumor is an essential prerequisite for successful eradication of cancer by T cells. Therefore, immunologic assays that demonstrate recognition of native tumor (tumor specific), such as that adapted for use in this study, may be more clinically relevant to assess T-cell responses after cancer vaccination, compared with assays that demonstrate recognition of tumor protein or peptide presented on appropriate antigen-presenting cells (antigen specific). Several reports in the literature indicate that endogenous antigens can be presented by MHC class II molecules in addition to MHC class I molecules on tumors (25, 31–35). Therefore, a tumor cell recognition assay can potentially detect both CD4\(^+\) and CD8\(^+\) T-cell responses.

In the present study, postvaccine, but not prevaccine, PBMCs from all 10 patients specifically reacted to autologous tumors by producing IFN\(\gamma\), GM-CSF, and/or TNF-\(\alpha\) in a class I- or II-associated manner, and either CD4\(^+\) or CD8\(^+\) T-cell subsets were sufficient to produce the response (Figs. 1–4). Moreover, tumor recognition assays potentially enable the detection of immune responses against the immunogen as well as other
antigens not represented in the vaccine. For example, in some cases, postvaccine T cells recognized autologous tumor but not autologous Id protein (UPN 3; Figs. 1, 3, 4, and 5). This observation is consistent with the possibility that immune responses against cryptic epitopes may have developed by epitope spreading, secondary to the inflammatory immune response initiated by Id-specific T cells (36–42). To further examine the possibility of epitope spreading, we are currently in the process of identifying the specific antigens recognized by the postvaccine, tumor-specific T cells by expression cloning methods.

Finally, although the use of autologous tumor cells as targets of a cytokine response in vitro (Figs. 1–4) may be highly desirable, it is acknowledged that primary tumor cells may not be generally accessible for all tumor types (e.g., solid tumors).

It is generally believed that optimal active specific immunization of cancer should generate both CD4+ and CD8+ T-cell responses against the targeted tumor. Our published preclinical studies demonstrated that incorporation of Id into liposomes along with IL-2 elicited both T-cell subsets and was more potent compared with a prototype Id-KLH vaccine (19). However, it is difficult to predict whether the human T-cell responses observed in the present study are different qualitatively or quantitatively from those produced by the Id-KLH + GM-CSF vaccine formulation, which was also administered in the clinical setting of first complete clinical response in a single-arm study (11). Comparison of different vaccine formulations would require the development of surrogate end points to assess vaccine potency because clinical outcome (disease-free survival) was promising.

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**Fig. 5** Postvaccine PBMCs specifically recognized autologous Id protein. Fresh (A–C) or cryopreserved PBMCs (D–F) were cultured with either medium alone or patient-specific Id protein or control isotype-matched irrelevant Id proteins. Cytokine production in the supernatants was measured by ELISA. A and D, IFNγ production. B and E, GM-CSF production. C and F, TNF-α production. Cryopreserved pre- and postvaccine PBMCs were tested in parallel in three patients (D–F).
in both studies. Furthermore, the adjuvants in each vaccine formulation may enhance the immune response via separate mechanisms of action. For example, GM-CSF may act via the afferent arm of the immune system by improving antigen presentation, whereas IL-2 may predominantly act via the efferent arm of the immune system by stimulating natural killer cells and T cells. Preclinical studies suggest that each of these adjuvants enhance the antitumor efficacy of vaccines and that the combination may be synergistic (43, 44). Future clinical studies should assess the relative benefit of these adjuvants when used alone or in combination.

Given the small number of patients, it is not possible to draw definitive conclusions regarding the correlation between immune responses and clinical outcome. The induction of a robust tumor-specific T-cell response was associated with a sustained second complete remission in patient 7 (Table 1; Fig. 4D). In contrast, despite the induction of tumor-specific T-cell responses in patients 3 and 8, these patients developed progressive disease at the end of the vaccination. Further characterization of the tumor-specific T cells, such as precursor frequency, memory T-cell induction, avidity, effector function such as granzyme B and perforin secretion, and Fas L expression, is probably needed. Additionally, one also needs to study various tumor escape mechanisms (45) that may be present in these patients. Evaluation of large numbers of patients with various immunologic assays may eventually identify surrogate end points that will facilitate the rapid comparison of different vaccine formulations in the future.

To conclude, this novel liposomal Id/IL-2 vaccine formulation induces sustained, tumor-specific CD4+ and CD8+ T-cell responses in FL patients and may serve as a model for liposomal delivery of other tumor antigens and infectious pathogens against which T-cell immunity is desirable (e.g., HIV). These data also provide the rationale for further streamlining the production of individualized tumor vaccines by directly extracting selected membrane proteins from the tumor cells and incorporating them into liposomes along with IL-2 or other potent cytokines.

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