ABSTRACT

B-cell lymphomas express tumor-specific immunoglobulin, the variable regions of which [idiotype (Id)] can serve as a target for active immunotherapy. Promising results have been obtained in clinical studies of Id vaccination using Id proteins. However, Id protein is laborious and time-consuming to produce. DNA vaccination is an attractive alternative for delivering Id vaccines, because Id DNA can be rapidly isolated by PCR techniques. DNA coding for immunogenicity of naked DNA Id vaccines in 12 patients with follicular B-cell lymphoma. The DNA encoded a chimeric immunoglobulin molecule containing variable heavy and light chain immunoglobulin sequences derived from each patient’s tumor, linked to the IgG2a and κ mouse immunoglobulin (MsIg) heavy- and light-chain constant regions chains, respectively. Patients in remission after chemotherapy received three monthly i.m. injections of the DNA in three dose escalation cohorts of four patients each (200, 600, and 1800 μg). After vaccination, 7 of 12 patients mounted either humoral (n = 4) or T-cell-proliferative (n = 4) responses to the MsIg component of the vaccine. In one patient, a T-cell response specific to autologous Id was also measured. Anti-Id antibodies were not detectable in any patient. A second series of vaccinations was then administered using a needle-free injection device (Biojector) to deliver 1800 μg both i.m. and intradermally (i.d.); 9 of 12 patients had humoral (n = 6) and/or T-cell (n = 4) responses to MsIg. Six of 12 patients exhibited humoral and/or T-cell anti-Id responses; yet, these were cross-reactive with Id proteins from other patient’s tumors. Subsequently, a third series of vaccinations was carried out using 500 μg of human granulocyte-macrophage colony-stimulating factor DNA mixed with 1800 μg of Id DNA. The proportion of patients responding to MsIg remained essentially unchanged (8 of 12), although humoral or T-cell responses were boosted in some cases. Throughout the study, no significant side effects or toxicities were observed. Despite the modest level of antitumor immune response in this study, DNA vaccine technology retains potential advantages in developing anti-Id immunotherapies. Additional studies are warranted to optimize vaccine dose, routes of administration, vector designs, and prime-boost strategies. These results will help guide the design of such future DNA vaccine trials.

INTRODUCTION

B-cell malignancies express a tumor-specific antigen, the immunoglobulin Id, which can serve as a target for immunotherapy (1). The Id antigenic determinants are located in the variable regions of the heavy and light chains of the tumor’s clonal immunoglobulin. These structures create targets that can be recognized by antibodies as well as by CD4+ and CD8+ T cells. Tumor-specific Id proteins can be isolated from lymphoma cells using hybridoma techniques (2). The protein can be rendered immunogenic by chemical coupling to a carrier protein such as KLH and by mixing with an immunological adjuvant or with dendritic cells. Vaccination of lymphoma patients with Id proteins has been shown to induce: (a) anti-Id immune responses that correlate with improved clinical outcomes (3, 4); (b) durable tumor regressions (5, 6); and (c) molecular complete remissions (7). However, several factors currently prohibit the effective application of Id vaccination to large numbers of lymphoma patients. The isolation of Id protein from each patient’s tumor is time consuming and expensive. Moreover, coinfected immunological adjuvants often contribute side effects at the local injection site.

DNA vaccination may represent a more efficient route to anti-Id immunotherapy. Vaccination of mice with plasmid DNA expression vectors (“naked DNA vaccination”) has been shown to induce protective immune responses against a variety of infectious diseases and tumors (8–10). Plasmid DNA constructs are technically easier and less time consuming to prepare than recombinant or hybridoma-derived proteins. This is especially important in the case of a customized vaccine approach. In addition, bacterial plasmid DNA possesses inherent immune adjuvant activity based on its content of unmethylated CpG immunostimulatory sequences (11) that interact with toll-like receptors in mammalian immune cells (12). Recent technological developments make a genetic approach to idiotype vaccination possible (13). PCR technology now allows for the rapid cloning of the variable (idiotype) regions from immunoglobulin genes (14, 15). Genetic vaccination with Id-encoding plasmid DNA has shown efficacy in several murine lymphoma models (16–19). Syrengelas et al. found that vaccination of mice with plasmid DNA-encoding tumor Id developed strong humoral anti-Id immune responses and were protected against subsequent tumor challenge at a rate comparable with that after vaccination with Id-KLH protein. The protection depended on the inclusion of foreign constant region immunoglobulin sequences (human) linked to the mouse variable regions, which were felt to provide a “carrier” function analogous to that provided by the KLH in the case of protein vaccination. Anti-Id immune responses were further enhanced by conjunction of DNA encoding GM-CSF (13, 16). These findings, and the development of a bicistronic immunoglobulin expression vector (20), formed the basis for the current study of DNA Id vaccination in humans.

We sought to explore the safety and immunogenicity of chimeric Id-encoding plasmid DNA vaccines with and without coinfected GM-CSF DNA in patients with lymphoma. Patient-specific plasmids were constructed to encode a chimeric immunoglobulin molecule consisting of tumor-specific variable (Id) regions linked to xenogeneic (murine) immunoglobulin constant regions, in a strategy analogous to that shown to be effective in our preclinical studies (16, 20, 21). We found...
that with the doses and routes of injection used, the Id DNA vaccines were safe and that the vector was immunogenic. The majority of patients developed humoral or T-cell immune responses to the xenogeneic (murine immunoglobulin) component of the vaccine, and some patients made responses to their autologous tumor Id.

MATERIALS AND METHODS

Patients. Eligible patients were adults with untreated stage III or IV follicular B-cell lymphoma (follicle center cell grades I and II by Revised European and American Lymphoma classification). All of the patients had a peripheral lymph node of at least 2 × 2 cm accessible to excisional biopsy for diagnostic confirmation and vaccine production. Monoclonal surface immunoglobulin expression was verified using flow cytometry. Patients received initial chemotherapy with six to eight cycles of cyclophosphamide, vincristine, and prednisone (CVP). Three patients (patients 7, 8, and 13) also received four cycles of cyclophosphamide, hydroxydaunomycin, vincristine, and prednisone for failure to achieve a MRD state after CVP. Clinical responses were classified according to the standardized response criteria of Cheson et al. (22). Vaccinations were initiated a minimum of 3 months after completion of chemotherapy. Pre vaccination patient evaluations included physical examination, complete blood counts, lymphocyte subset analysis, serum chemistries, rheumatoid factor, anti-dsDNA antibodies, CT scans of the chest, abdomen, and pelvis, and bilateral iliac crest bone marrow biopsies. On the day of each vaccination, and at 1 and 3 months after each series of vaccinations, laboratory studies included complete blood counts, serum chemistries, rheumatoid factor, anti-dsDNA antibodies, and humoral and cellular immune responses to Mls Ig and tumor Id. Three months after each vaccination series, CT scans and bone marrow biopsies were repeated; and subsequent follow-up examinations, performed every 3 months, included physical examination, complete blood counts, and serum chemistries. Repeat CT scans were performed every 6 months for the first 2 years and annually thereafter, or as clinically indicated. The prevaccine tumor status of patients was classified as NED, MRD, or RD, as previously described (14, 15), but incorporating Sfi restriction sites (20). The amplified, Sfi-digested fragments of the heavy and light chains were then ligated into plasmid VCL-1632 to yield plasmids VCL-1642.XXX (Fig. 1A). Plasmid VCL-1632 is a pUC18-derived kanamycin-selectable plasmid containing the CMV promoter followed by the murine k constant region sequence, a translational enhancer, the murine IgG2a heavy-chain sequence, and a translation terminator. The completed plasmids, thus, expressed a bicistronic RNA encoding a chimeric human-murine immunoglobulin containing tumor-specific variable regions linked to murine constant regions. Proper insertion and orientation of the immunoglobulin domains was verified in individual clones by restriction digest mapping followed by DNA sequence analysis. To confirm functional expression of the encoded immunoglobulin, purified plasmids were used to transfect VM92 (murine melanoma) cells, and the supernatants were analyzed by ELISA and Western blot to detect full-length, functional immunoreactive chimeric immunoglobulin (data not shown). The best-producing clones were then chosen for vaccine production. Plasmid VCL-1723 encoding human GM-CSF (Fig. 1B) has been described previously (23). The biological activity of plasmid VCL-1723 was confirmed by the ability of supernatants from VCL-1723-transfected VM92 cells to promote the growth of GM-CSF-dependent TF-1 cells (24). Large-scale production of clinical-grade plasmid DNAs, along with purification and quality assurance analyses, were performed as previously described (25, 26). Vaccines were formulated to contain 200, 600, or 1800 μg of Id DNA or 500 μg of GM-CSF DNA in 1.0 ml of sterile PBS, and were stored frozen until use.

Tumor Id Protein Production. Native tumor Id proteins were isolated for each patient using the rescue hybridoma method (3, 4). These proteins were used for in vitro assessment of anti-Id immune responses. Id proteins were purified by affinity chromatography as described previously (3).

Vaccine Treatments. Patients received three series of vaccinations over the course of the study as summarized in Fig. 2. Series 1 vaccinations represented a dose-escalation phase, in which three groups of four patients each received three monthly i.m. (deltoid) injections, by conventional needle and syringe, of 200, 600, or 1800 μg of murine IgG2a and κ constant regions, which served as a foreign carrier. B, plasmid vector encoding human GM-CSF used for conjunction with DNA encoding chimeric tumor Id.
Tumor biopsy, Chemotherapy, Tumor Id DNA cloning and GMP production

Series #1 vaccinations
Dose escalation Id DNA (200, 600, or 1800 μg)
Needle & syringe, i.m.
Monthly x 3

17 months

Series #2 vaccinations
1800 μg Id DNA
Biojector; 80% i.m., 20% i.d.
Monthly x 3

14 months

Series #3 vaccinations
1800 μg Id DNA + 500 μg GM-CSF DNA
Biojector; 80% i.m., 20% i.d.
Monthly x 3

Fig. 2. Clinical trial schema. The numbers of months indicate the time intervals between initiation of the different vaccination series.

response was interpreted as positive (+) when incorporation of more than twice background (mean ± 3 SD) was observed on two or more occasions. Responses were designated ‘+’; when incorporation of more than twice background and pre-vaccine baseline was observed on only a single occasion postvaccine. Tumor-specific cytotoxicity and cytokine release by PBMCs cocultured with tumor cells were measured as described previously (6, 7).

Humoral Immune Response Assessments. Sera of all patients were analyzed for IgG anti-Id antibodies by ELISA. Tumor-derived Id proteins were of either IgM or IgG subclass depending on the type expressed by the tumor. Autologous Tumor Id proteins or irrelevant IgM Id proteins were captured onto microtiter plates coated with goat anti-human IgM (Biosource International, Camarillo, CA). When the tumor Id was an IgG, a recombinant Id protein was constructed containing the tumor Id linked to human IgG3 constant regions, and captured onto the plate with goat anti-human IgG3 (Biosource). The need to detect bound IgG antibodies necessitated isolation of the variable region/Id sequences from commonly used IgG constant region sequences to minimize background in the assay. The IgG3 isotype was chosen for target Id construction given its generally minor contribution to humoral responses. Pre-vaccine and post-vaccine sera were serially diluted and allowed to bind to the target Id proteins. The reagents used to detect bound antibodies depended on the isotype of the target Id molecule. When the Id protein was an IgM, bound anti-Id antibodies of the IgG subclass were detected using polyclonal goat antihuman IgG (Biosource) coupled to HRP. When the target Id protein was an IgG3, bound anti-Id antibodies were detected with a cocktail of mouse monoclonal antihuman IgG1, IgG2, and IgG4 antibodies conjugated to HRP (Southern Biotech, Birmingham, AL). In each case, HRP-conjugated antibodies to the immunoglobulin light chain (κ or λ) opposite to that of the tumor Id was used to detect serum antibodies bound to the target Id. A response was considered positive (+) when a 4-fold increase in anti-Id titer was found compared with both the pre-vaccine serum and the isotype-matched irrelevant Id proteins used as specificity controls. Antibody responses to murine IgG2a/κ were measured by coating microtiter plates with mouse antibody S1C5 followed by incubation with diluted sera as above and detection using polyclonal goat antihuman IgG-HRP. A response was considered positive (+) when a 4-fold increase in anti-Id titer was found compared with the pre-vaccine serum.

Safety Assessments. Patients were observed for adverse effects for at least 1 h after each injection, and, thereafter, a diary was kept to record any local injections site reactions or other side effects. Serum was tested for rheumatoid factor and anti-dsDNA antibodies at baseline and 3 months after each vaccination series. For Series 3, evaluations also included serum creatine phosphokinase before, and 1 month after, each vaccination. Mouse IgG2a/κ in serum was measured by ELISA (sensitivity of assay <0.1 μg/ml), using S1C5 as a standard. Serum GM-CSF was measured by ELISA (R&D Systems, Minneapolis, MN). Serum antibodies against GM-CSF were measured using a sandwich ELISA technique. Recombinant human GM-CSF (Immunex, Seattle, WA) was used to coat plates, to which serially diluted patient sera were added. Bound antibodies were detected using biotinylated GM-CSF followed by streptavidin-HRP (Vector Labs, Burlingame, CA). Sensitivity of the assay was <0.05 μg/ml.

RESULTS

Dose Escalation of Lymphoma Id DNA Vaccine. This study was designed primarily to investigate the safety and immunogenicity of Id DNA vaccines in humans. The doses of DNA were chosen based on previous work in mice in which 10–100 μg were sufficient to provide protective immunity (16). Dose escalation was carried out in three groups of four patients each, with the range of doses used being 200, 600, or 1800 μg. Vaccinations given during this initial dose escalation phase of the study were designated Series 1 (Fig. 2). The prevaccine tumor status, starting vaccine dosage levels, peripheral blood CD4+ T cell counts, and immune response data for each patient are shown in Table 1. CD4+ T-cell counts were notably depressed in these patients postchemotherapy and remained so in most cases throughout the duration of the study (mean, 371 ± 119/μl; normal range, 540-1660/μl). At vaccine initiation, four patients had NED, four had MRD, and four had residual tumor in lymph nodes and/or bone marrow. The plasmid DNA was injected i.m. by needle and syringe into the deltoid region. No significant side effects or toxicities were noted other than those expected from routine i.m. injections. A minority of patients had mild local injection site tenderness lasting 1–2 days.

All 12 of the patients completed the three vaccinations and were evaluable for immune responses. Seven patients (58%) mounted immune responses to the MsIg component encoded by the DNA vaccine. There were four humoral responses, four T-cell proliferative responses, and one patient with both humoral and T-cell responses (Tables 1 and 2). Immune responses were observed in a similar proportion of patients in each dose cohort: 2/4, 3/4, and 2/4 patients in the 200, 600, and 1800 μg dose cohorts, respectively (Table 1). An example of a T-cell response against MsIg observed in patient 5 is shown in Fig. 3. In this case, the proliferation of PBMCs to MsIg was concentration dependent and was seen on multiple occasions after vaccination (data not shown). A more modest proliferative response to tumor Id was also observed at this particular time point. One other patient (patient 12), treated at the highest (1800 μg) dose level, developed a reproducible tumor Id-specific T-cell proliferative response after vaccination (Fig. 4).

Repeat Vaccinations with 1800-μg Dose Via Biojector. Given the relatively low starting doses administered during dose escalation, the low rate of anti-Id immune responses, and the demonstrated safety of the 1800 μg dose, we chose to revaccinate all of the eligible patients with this highest dose in an attempt to boost immunity. These Series 2 vaccinations began 17 months after the initiation of Series 1, after which time, adequate safety data on the 1800-μg dose cohort were available and regulatory approval had been granted. The median interval between completion of Series 1 and the start of Series 2 vaccinations was 7 months (range, 3–15 months). Patient 2 had withdrawn from the study shortly after Series 1 because of progressive disease requiring standard therapy and was replaced by patient 13. This patient and three others (patients 3, 8, and 12) had evidence of disease progression at the time of Series 2. For Series 2 vaccinations, we used a needle-free jet injection device (Biojector) to deliver 80% of the dose i.m., and 20% i.d. Prior studies had indicated that i.m. jet injection delivery of a DNA vaccine could provide superior immune.
responses over conventional i.m. injection using needle and syringe (28, 29). It was assumed that the use of the Biojector would increase the immunogenicity of any given dose of DNA; therefore, only the previously established fixed dose of 1800 μg was used in subsequent portions of the study. The incidence and severity of i.m. injection site reactions in Series 2 was indistinguishable from that seen with needle-and-syringe injections in Series 1. In addition, no appreciable local reactions were observed at the sites of i.d. inoculation. After Series 2 vaccinations, there were a total of 12 immune responses against mouse immunoglobulin detected in 9 patients (6 humoral, 6 T cell, 3 both humoral and T cell; Tables 1 and 2). Four of these nine immune responders had failed to respond after Series 1 vaccinations, whereas both humoral and T cell; Tables 1 and 2). Four of these nine immune responders had failed to respond after Series 1 vaccinations, whereas four other patients had "nonspecific" T-cell anti-Id responses and two patients had nonspecific humoral anti-Id responses in which cross-reactivity to Id proteins from other patients’ tumors was observed.

### Repeat Vaccinations Using Coinjected GM-CSF DNA.

Coinjection of plasmids encoding cytokines such as GM-CSF has been shown to augment protective immunity induced by DNA vaccines (9, 13, 16, 30). We thus chose to administer a third round of vaccinations (Series 3) to these same patients using a coinjected plasmid encoding human GM-CSF (VCL-1723, Fig. 1B) in an attempt to boost the immune response to tumor Id. Vaccines consisted of 1800 μg of the chimeric Id plasmid DNA admixed with 500 μg of GM-CSF DNA delivered via Biojector as in Series 2. Series 3 vaccinations were begun 14 months after the initiation of Series 2, after which time adequate safety data on Series 2 vaccinations was available and regulatory approval had been granted. The median interval between completion of Series 2 and the start of Series 3 vaccinations was 10 months.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Series no.</th>
<th>Dosage in series no. 1</th>
<th>Prevaccine tumor status</th>
<th>CD4 count</th>
<th>Antibody response</th>
<th>T-cell response</th>
<th>Current status, time since (initial) chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>200 μg</td>
<td>NED</td>
<td>280</td>
<td>+/−</td>
<td>NED</td>
<td>NED, 52 mo</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>200 μg</td>
<td>MRD</td>
<td>ND</td>
<td>−/−</td>
<td>NED after subsequent HDCT + ASCT, 52 mo</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>200 μg</td>
<td>NED</td>
<td>530</td>
<td>+/−</td>
<td>PD, 47 mo</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>200 μg</td>
<td>RD</td>
<td>490</td>
<td>−/−</td>
<td>NED, 51 mo</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>600 μg</td>
<td>NED</td>
<td>190</td>
<td>+/−</td>
<td>NED, 49 mo</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>600 μg</td>
<td>RD</td>
<td>180</td>
<td>−/−</td>
<td>SD, 45 mo</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>600 μg</td>
<td>MRD</td>
<td>330</td>
<td>+/−</td>
<td>SD, 44 mo</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>600 μg</td>
<td>RD</td>
<td>428</td>
<td>+/−</td>
<td>Expired with PD, 34 mo</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>1800 μg</td>
<td>RD</td>
<td>506</td>
<td>+/−</td>
<td>SD, 40 mo</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>1800 μg</td>
<td>RD</td>
<td>441</td>
<td>+/−</td>
<td>NED, 37 mo</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>1800 μg</td>
<td>MRD</td>
<td>285</td>
<td>+/−</td>
<td>NED, 39 mo</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>1800 μg</td>
<td>MRD</td>
<td>424</td>
<td>+/−</td>
<td>SD, 39 mo</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>None</td>
<td>n/a</td>
<td>372</td>
<td>+/−</td>
<td>Rituximab, 26 mo</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
<td>328</td>
<td>+/−</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Patient characteristics, immune responses, and long-term follow-up

* Scoring of immune responses is as follows. Humoral responses: +, ≥2 dilutions above background and baseline and MsIg- or Id-specific; +/−, ≥1 dilution but <2 dilutions above background and baseline; −, <1 dilution above background and baseline; and ns, response not specific for patient’s own idiotype. Cellular responses: +, ≥2 times cpm above background and baseline at two or more time points and MsIg- or Id-specific; +/−, ≥2 times cpm above background and baseline at one time point; −, ≥2 times cpm above background and baseline at no time point; ns, response not specific for patient’s own idiotype.

MsIg, mouse IgG2a/c; NED, no clinical evidence of disease; MRD, minimal residual disease; RD, residual disease; PD, progressive disease; n/a, not applicable; SD, stable disease; HDCT + ASCT, high-dose chemotherapy and autologous stem cell transplantation; ND, not done.
concentrations. Proliferation was measured on day 5 by \[^{3}H\] thymidine incorporation. Results represent means ± SD of quadruplicate values.

Fig. 3. T-cell proliferative response to MsIg and tumor Id after DNA vaccination (Patient 5, 600-μg cohort). Postvaccine PBMCs were incubated with medium alone or tumor Id protein, irrelevant Id proteins, or murine IgG2a/c (MsIg) at the indicated concentrations. Proliferation was measured on day 5 by \[^{3}H\] thymidine incorporation. Results represent means ± SD of quadruplicate values.

Fig. 4. Cellular proliferative response to tumor Id after DNA vaccination (Patient 12, Series 1). Postvaccine PBMCs were incubated with medium alone, tumor Id protein, or irrelevant Id protein at 100 μg/ml. Proliferation was measured on day 5 by \[^{3}H\] thymidine incorporation. Results represent means ± SD of quadruplicate values.

(range, 4–12 months). By the time Series 3 vaccinations were initiated, 5 of the 12 patients had evidence of tumor progression (Table 1). The incidence and severity of i.m. injection site reactions in Series 3 were indistinguishable from that seen in Series 2, and no systemic toxicity was noted. Notably, no obvious local inflammatory reactions were observed at the sites of i.d. inoculation. After Series 3 vaccinations, there were a total of 11 immune responses against mouse immunoglobulin detected in eight patients (7 humoral, 4 T cell, 3 both humoral and T cell; Tables 1 and 2). Seven of the nine previously responding patients continued to display humoral or T-cell reactivity toward mouse immunoglobulin, and there was one new responder. Patient 12, who had new evidence of tumor involving bowel and skin, no longer had a specific T-cell proliferative response against autologous tumor Id. No other patient showed humoral or T-cell reactivity toward Id that was tumor-specific, although five patients had nonspecific anti-Id responses.

In some instances, immune responses to MsIg were boosted by Series 3 injections. Fig. 5 shows the T-cell proliferative response for patient 13 at baseline and after Series 2 and Series 3 immunizations. The data indicate significant enhancement of the response after the repeat series of vaccinations. Boosting of the humoral immune response to MsIg with the sequential series of DNA vaccinations is shown in Fig. 6. After Series 1, patient 5 showed no evidence of a humoral response to MsIg. However, after Series 2, a significant response was detectable, with further boosting of the response after Series 3 injections.

During the last phase of this clinical trial (Series 3), we performed additional studies to seek evidence of evoked antitumor immunity by the DNA Id vaccine. Autologous tumor cells were cocultured with fresh PBMCs both before and 1 month after Series 3 injections, as described previously (7). Supernatants of these cocultures were assayed for released tumor necrosis factor α and GM-CSF, yet no consistent tumor-specific pattern of cytokine release was observed (data not shown). We also investigated the cytotoxic activity of pre- and postvaccine PBMCs restimulated with autologous CD40 ligand-activated tumor cells toward cryopreserved tumor cells (6, 7). No tumor-specific lysis was exhibited in any of the four cases tested (data not shown).

**Safety of DNA Vaccinations.** No acute or long-term toxicities of the DNA vaccination maneuvers used have been identified in over 4 years of follow-up in this patient cohort. Five patients (patients 8, 9, 11, 12, and 13) developed modest, transient elevations of serum rheumatoid factor between Series 2 and 3 vaccinations (range, 21–58; normal level, <20). Patient 11 had a slightly elevated level of 26 at prevaccine baseline. This level peaked at 58 just prior to Series 2, subsided just prior to Series 3, and thereafter fell to within normal range. However, no patient developed clinical rheumatological manifestations or other symptoms or signs of autoimmune disease. Cases in which serum rheumatoid factors were elevated showed no obvious correlation to those in whom nonspecific anti-Id responses were noted.

**Table 2. Summary of immune responses in vaccination series 1–3**

| Antibody responders | T-cell responders | Total responders anti-MsIg
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MsIg</td>
<td>Id</td>
<td>MsIg</td>
</tr>
<tr>
<td>Total series 1</td>
<td>4/12</td>
<td>0/12</td>
</tr>
<tr>
<td>Dose escalation</td>
<td>4/12</td>
<td>1/12</td>
</tr>
<tr>
<td>Series 2 1800 μg</td>
<td>6/12</td>
<td>2ns/12</td>
</tr>
<tr>
<td>Bijector</td>
<td>4/12</td>
<td>4ns/12</td>
</tr>
<tr>
<td>80% i.m., 20% i.d.</td>
<td>1/12</td>
<td>1/12</td>
</tr>
<tr>
<td>Series 3</td>
<td>7/12</td>
<td>3ns/12</td>
</tr>
<tr>
<td>As Series 2, plus GM-CSF DNA (500 μg)</td>
<td>7/12</td>
<td>3ns/12</td>
</tr>
</tbody>
</table>

a MsIg, mouse IgG2a/c; Id, autologous tumor idiotype; ns, not specific for autologous Id.

b Specific for autologous Id.

Fig. 5. Boosting of the cellular immune response to MsIg after repeat series of DNA vaccinations. Patient 13 developed a modest PBMC proliferative response to MsIg after Series 2 vaccinations (This patient did not receive Series 1). An enhanced proliferative response after a booster series of vaccinations (Series 3) is shown.
DNA VACCINATION FOR LYMPHOMA

DISCUSSION

In this study, we were able to evaluate the immunogenicity of a plasmid DNA vaccine encoding a clinically relevant tumor antigen, Id, simultaneously with that of a xenogeneic, and presumably more immunogenic carrier protein, murine immunoglobulin. The plasmid DNA was administered at relatively low doses (200, 600, or 1800 μg) during the dose-escalation phase of the study (Series 1). Notably, even doses as low as 200 μg were able to stimulate humoral immunity against the murine immunoglobulin encoded by the DNA immunogen. Most patients mounted immune responses against this foreign component of the vaccine. The proportion of patients responding to the mouse immunoglobulin after Series 1, 2, and 3 vaccinations was 58% (7/12), 75% (9/12), and 67% (8/12), respectively. Eleven of the 13 patients displayed immune responsiveness to mouse immunoglobulin. The proportion of patients responding to the murine immunoglobulin encoded by the DNA immunogen. Most patients mounted immune responses against this foreign component of the vaccine. The proportion of patients responding to the mouse immunoglobulin after Series 1, 2, and 3 vaccinations was 58% (7/12), 75% (9/12), and 67% (8/12), respectively. Eleven of the 13 patients displayed immune responsiveness to mouse immunoglobulin. The proportion of patients responding to the murine immunoglobulin encoded by the DNA immunogen. Most patients mounted immune responses against this foreign component of the vaccine. The proportion of patients responding to the mouse immunoglobulin after Series 1, 2, and 3 vaccinations was 58% (7/12), 75% (9/12), and 67% (8/12), respectively. Eleven of the 13 patients displayed immune responsiveness to mouse immunoglobulin. The proportion of patients responding to the murine immunoglobulin encoded by the DNA immunogen. Most patients mounted immune responses against this foreign component of the vaccine. The proportion of patients responding to the mouse immunoglobulin after Series 2 and was further boosted after Series 3 injections. Binding of antibodies in serum obtained at different time points is represented by raw absorbance readings (O.D.) obtained simultaneously by ELISA. Sets of arrows on X axis, monthly vaccinations in Series 1, 2, and 3.

Fig. 6. Boosting of the humoral immune response to MsIg with sequential series of DNA vaccinations. Patient 5 failed to develop a humoral response to MsIg after Series 1 vaccinations. However, a response was detectable after Series 2 and was further boosted after Series 3 vaccinations. Binding of antibodies in serum obtained at different time points is represented by raw absorbance readings (O.D.) obtained simultaneously by ELISA. Sets of arrows on X axis, monthly vaccinations in Series 1, 2, and 3.

Long-Term Clinical Follow-Up. Given that many patients had measurable tumor at different time points during this study, we had the opportunity to evaluate patients for tumor regression responses. Patient 4 had residual tumor in the bone marrow on initiation of Series 1 vaccinations. Bone marrow involvement was diminished after Series 2, and was not detected after Series 3 vaccinations, and this patient remains in complete clinical remission 49 months after completing chemotherapy. No other objective tumor regressions were observed. At a median follow-up of 44 months postchemotherapy, four patients remain without evidence of tumor, and four patients have stable RD. Five patients have developed progressive disease, and three of these (patients 2, 8, and 13) required additional therapies. Patient 8 died with progressive tumor because of complications after high-dose chemotherapy given in preparation for stem cell transplantation.

It is important to view our results in the context of the limited data available on DNA vaccination in humans. In early trials of DNA vaccines encoding HIV or influenza antigens, the induction of antibodies, T-cell proliferation, or CTLs was reported, although most often in only a minority of patients (31). More recently, i.m. injection of DNA encoding the malarial circumsporozoite protein has been shown to induce CD8+ CTLs in a majority of naïve, healthy subjects (32). CTL responses were detectable with doses as low as 20 or 100 μg but were more frequent at higher (500 and 2500 μg) doses. Despite the induction of robust CTL responses, humoral responses to circumsporozoite protein were not detectable (33). Particle-mediated delivery of a HBsAg DNA vaccine has now been shown to efficiently induce protective antibody titers and both CD4+ and CD8+ T-cell responses in healthy volunteers (34). Thus, the immunogenicity of DNA encoding foreign proteins from infectious pathogens has been demonstrated repeatedly.

5 J. Timmerman and R. Levy, unpublished observations.
In contrast, eliciting antitumor immunity in cancer patients using DNA vaccines has proved more difficult. Hawkins et al. (15, 35) constructed plasmid DNA encoding lymphoma Id as single-chain Fv fragments (heavy and light chain variable regions joined by a flexible linker) from 10 patients with low-grade B-cell lymphoma. However, at doses of 100–500 μg delivered i.m., they failed to find evidence of anti-Id immunity. Investigators at the National Cancer Institute Surgery Branch performed a study of DNA vaccination against the melanoma differentiation antigen gp100 (36). Twenty-three patients with metastatic melanoma were treated using doses of 100–1000 μg divided between two i.m. and i.d. sites, either with or without systemic interleukin 2. In this trial, no clear signs of anti-gp100 immunity or objective tumor regressions were noted. White and Conroy (31) performed vaccination of 17 patients with metastatic colorectal carcinoma using a dual expression plasmid encoding the self tumor antigen carcinoembryonic antigen along with HBsAg as a control. Plasmid DNA was injected i.m. at doses of 100, 300, 1000, or 2000 μg. Although dose-dependent induction of anti-HbsAg antibodies was noted, no antibody or T-cell responses against carcinoembryonic antigen were detected. This latter result is analogous to the present study, in which immune responses against the foreign component of the vaccine were frequently induced, yet responses to the relevant tumor antigen were undetectable or weak (Figs. 3 and 4). Thus, little evidence of antitumor immunity has been demonstrated using first generation tumor antigen DNA vaccines.

The results of tumor antigen DNA vaccine approaches might be improved by optimization of key variables such as dosage, route, vector design, and boosting strategies (37). Most simply, elevated doses may prove to be more effective. Whereas the doses used in our study (200–1800 μg) were within the range of doses found to be immunogenic in humans using infectious disease antigens, on a per weight basis they are still 10–100 fold lower than those which provide optimum tumor protection in mice (9, 16). At the time our study was initiated, there were no published reports describing the safety or immunogenicity of plasmid DNA vaccines in humans; therefore, the appropriate dose range was not known, and the range of doses chosen reflected primarily safety concerns. Dose dependence of immune responses has now been demonstrated in numerous preclinical (37) and human studies (31, 32, 37). Barouch et al. found that large doses of DNA (10 mg) were required to provide protection of primates against a simian immunodeficiency virus challenge.

The method and route of injection also greatly influences the efficacy of DNA vaccines. The i.m. needle injection appears to be the least efficient, whereas needle-free jet injection (Biojector) can improve the immunogenicity of a given DNA vaccine dose (28, 29). Delivery of DNA to the epidermis by gold particle bombardment also prove the immunogenicity of a given DNA vaccine dose (28, 29). Despite the unsatisfactory antitumor immune responses observed in the present study, DNA vaccination remains an attractive platform for the further development of Id vaccines. Promising clinical activity of protein and dendritic cell-based Id vaccines has already been demonstrated (3–7), yet the broad application of patient-specific Id vaccines to the treatment of B-cell malignancies may necessitate more simplified vaccine production techniques. DNA vaccine technology has the potential to allow the streamlining of customized vaccine manufacturing, along with unique opportunities to manipulate the antigenic sequences to include additional immunogenic epitopes, cellular targeting sequences, and immunostimulatory motifs. Additional studies are warranted to optimize vaccine dosage, vector designs, routes of administration, and prime-boost sequences. Our results will help guide the design of such future Id DNA vaccine trials.

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