A vaccine directed to B cells and produced by cell-free protein synthesis generates potent antilymphoma immunity

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Contributed by Ronald Levy, July 10, 2012 (sent for review May 26, 2012)

Clinical studies of idiotype (Id) vaccination in patients with lymphoma have established a correlation between the induced anti-Id antibody responses and favorable clinical outcomes. To streamline the production of an Id vaccine, we engineered a small diabody (Db) molecule containing both a B-cell–targeting moiety (anti-CD19) and a lymphoma Id. This molecule (αCD19-Id) was designed to penetrate lymph nodes and bind to noncognate B cells to form an antigen presentation array. Indeed, the αCD19-Id molecule accumulated on B cells in vivo after s.c. administration. These noncognate B cells, decorated with the diabody, could then stimulate the more rare Id-specific B cells. Peptide epitopes present in the diabody linker augmented the response by activating CD4+ helper T cells. Consequently, the αCD19-Id molecule induced a robust Id-specific antibody response and protected animals from tumor challenge. Such diabodies are produced in a cell-free protein expression system within hours of amplification of the specific Ig genes from the B-cell tumor. This customized product can now be available to vaccinate patients before they receive other, potentially immunosuppressive, therapies.

Idiotype | tumor-specific antigen | bispecific antibody fragments

Idiotype (Id), the unique Ig molecule of each lymphoma tumor, is a good target for the immune system. Passively administered monoclonal antibodies (mAbs) against this target are effective in therapy (1). Furthermore, studies of Id vaccination had suggested a correlation between induced anti-Id antibody responses and progression-free survival and overall survival of patients (2–4). Despite these encouraging results, phase III trials have not established a clinical benefit from Id vaccination, except for a possible subset of patients who have prolonged remissions after initial chemotherapy (5–7). One possible problem may have been the chemical conjugation of Id to the carrier protein, keyhole limpet hemocyanin (KLH). Antigenic determinants on the Id could have been damaged in this process (8). Recombinant vaccines that do not require chemical conjugation may lead to improved immunogenicity and clinical outcomes.

Recent studies on antigen (Ag) acquisition by B cells have provided new insights for vaccine design. The majority of B cells reside in follicles within secondary lymphoid organs. Foreign Ags in the form of immune complexes are transported into lymph node follicles by subcapsular sinus macrophages (9–11), and into spleen follicles by marginal zone B cells (12). In the follicles, nonspecific B cells retain immune complexes on their cell surfaces. Some complexes are transferred to follicular dendritic cells (9–11), whereas others may directly cross-link the Ag-specific receptors (BCRs) on cognate B cells (10, 11). These roles played by noncognate B cells in the generation of specific antibody responses were previously not appreciated. In addition to forming immune complexes that facilitate entering the follicles and presenting on the cell surface, foreign Ags may also be endocytosed, processed, and presented as peptides that activate CD4+ T cells, which in turn, provide costimulation to cognate B cells. These attributes argue for the use of foreign carrier proteins such as KLH to help stimulate antibody responses against self-Ags that do not form immune complexes. However, chemical conjugation has been shown to reduce vaccine potency (8). Recombinant Id vaccines may offer distinct advantages because they can be produced with built-in carrier moieties.

It was recently discovered that small molecules (<70 kDa) can enter follicles more efficiently through specialized conduits (13, 14). We therefore designed a recombinant vaccine below this size limit. To provide cell surface anchorage for Ag retention and presentation, we delivered the vaccine to noncognate B cells within the follicle by targeting to CD19, a B-cell–specific molecule (15). We created a bispecific diabody (Db), containing the variable regions of a rat anti-mouse CD19 mAb and those of the 38C13 mouse B-cell lymphoma Id [αCD19-Id, molecular weight: 52 kDa]. We envisioned that the αCD19-Id would form a “lawn” of Ags on the surface of follicular B cells, where they could cross-link the BCR of the rare Ag-specific B cell among them. Furthermore, coligation of the BCR with CD19 could result in synergistic activation of the specific B cells (15). Nonsynonymous sequences, such as the rat variable regions in the Db, might help by activating CD4+ T cells (Fig. 1).

We used an in vitro (cell-free) protein synthesis (CFPS) system for mammalian proteins that can assemble intrachain disulfide bonds (16, 17). The reaction contains the DNA template for each polypeptide chain, an energy source, substrates, and cellular machinery from Escherichia coli that can carry out both transcription and translation. A small reaction can produce protein sufficient for vaccination in a matter of hours, as opposed to the usual methods of mammalian cell protein production that take several weeks. We produced and screened several structural variants of αCD19-Id. The most active form was then used for in vivo studies.

Results

Diabody Design, Production, and Initial Characterizations. αCD19-Id is a heterodimer of noncovalently associated polypeptides containing the variable regions of 38C13 and anti-CD19, separated by Gly3Ser linkers (Fig. 2A). We produced four different αCD19-Ids with the respective variable domains in different orientations (Fig. 2A and Fig. S1). The only polypeptides that incorporate a radio labeled amino acid are those encoded by the supplied templates. This labeling allows quantification and SDS/PAGE autoradiography without purification, thus expediting screening


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.orglookup/suppl/doi:10.1073/pnas.1211018109/DSpublished.

14526–14531 | PNAS | September 4, 2012 | vol. 109 | no. 36

www.pnas.org/cgi/doi/10.1073/pnas.1211018109
of various constructs. The “open” feature of CFPS also allowed us to adjust the relative amounts of the two template plasmids to ensure a 1:1 chain ratio in each Db heterodimer. The Db proteins were screened by flow cytometry for appropriate binding activities (Fig. 2B). Bispecific binding was determined using a target cell (A20) that expresses surface CD19 and a detector consisting of an anti-38C13 Id mAb. As a negative control cell we used a subclone of the A20 cell line that had lost cell surface expression of CD19 (A20/CD19<sup>−/−</sup>) (18). Among these four products, αCD19-Id-1 showed the best bispecific binding activity (Fig. 2B). We made a negative control Db that had the variable regions of a rat mAb of irrelevant specificity (RatFv-Id) (Fig. S1). Both Dbs were confirmed to be single species heterodimers by SDS/PAGE and size-exclusion (SE)-HPLC (Fig. S2 A and B), and were used for subsequent studies.

**αCD19-Id Localized to B Cells in Vivo.** We injected mice intradermally (i.d.) with fluorophore-labeled Dbs and analyzed cells from the draining lymph nodes by flow cytometry. αCD19-Id, but not RatFv-Id, was retained specifically on B cells (B220<sup>+</sup> population) but not on T cells (CD3<sup>+</sup> population) (Fig. 3A), and this occurred as early as 2.5 h after injection (Fig. S3A). This rapid accumulation is similar to a report of a small Ag (turkey egg lysozyme, 14 kDa) that traveled through conduits into follicles (14). The efficiency of B-cell targeting was even more apparent when cells from the spleen and blood were analyzed 2 h after i.v. injection of αCD19-Id. Again, we found binding of the specific Db to B cells, but not to T cells (CD3<sup>+</sup>), monocytes, macrophages, or granulocytes (CD11b<sup>+</sup> and F4/80<sup>+</sup>) (Fig. 3B and Fig. S3 B and C).

**Id-Specific BCR Activation by αCD19-Id-Decorated B Cells.** For this test we constructed an Id-specific B cell (A20/α38BCR) by transfecting the A20 cell line to express a membrane-anchored form of the anti-Id antibody (Fig. S4). We demonstrated that splenic B cells recovered from animals injected with αCD19-Id-1 (Fig. 4A), or A20 cells decorated with αCD19-Id in vitro (Fig. S5A), could trigger the phosphorylation of intracellular BCR pathway signaling molecules in Id-specific B cells. These signals peaked at 20 min and declined gradually over 30–60 min after the stimulator and responder cells came in contact (Fig. 4A and B). This stimulation did not occur in the negative control cell line, native A20, that lacked the specific anti-Id BCR. We also found that A20 cells decorated with αCD19-Id induced a stronger activation signal than that induced by an equal amount of free αCD19-Id, and reached a level to that induced by the pentameric 38C13 IgM protein (Fig. S5B). Another way αCD19-Id could stimulate an Id-specific B cell is by cross-linking its BCR to its CD19 surface molecule. In fact, αCD19-Id induced phosphorylation of phosphatidylinositol 3-kinase (PI3K), a signaling molecule directly downstream of CD19 (15), as well as the extracellular signal-regulated protein kinase (ERK) (Fig. 4C). Neither the negative control RatFv-Id (Fig. 4C) nor an anti-CD19 mAb induced such phosphorylation.

**Id-Specific B Cells Captured αCD19-Id from Db-Decorated B Cells and Internalized the Vaccine Molecule.** Ag-specific B cells need to internalize their cognate Ag for processing and presentation to receive CD4<sup>+</sup> T-cell help. Splenic B cells from mice injected with fluorophore-labeled αCD19-Id (Fig. 5A), or A20 cells decorated with fluorophore-labeled αCD19-Id in vitro (Fig. S6), could transfer the Db to A20/α38BCR cells, but not to A20 cells lacking the specific BCR. We also confirmed that αCD19-Id was internalized by A20/α38BCR cells using confocal microscopy (Fig. 5B).

**αCD19-Id Induced Both an Id-Specific Antibody Response and a Db-Specific T-Cell Response.** αCD19-Id induced a robust Id-specific IgG response, comparable to that induced by 38C13-KLH. By contrast, immunization with RatFv-Id, αCD19 + 38C13, or αCD19-Av-38C13 failed to induce a significant response (Fig. 6A). Whereas both groups of antibody responding mice made predominantly anti-Id IgG1, αCD19-Id induced a slightly higher percentage of anti-Id IgG2 than that induced by 38C13-KLH (33.8 ± 6.2% vs. 22.4 ± 2.8%, as mean ± SD).

The anti-Id antibody response induced by 38C13-KLH requires CD4<sup>+</sup> T cells (8). That was also the case for αCD19-Id. Depletion of CD4<sup>+</sup> T cells from animals before vaccination with the Db dramatically reduced the anti-Id IgG responses (from 77, 50, and 20 μg/mL to 6, 0, and 0 μg/mL serum). Lymphocytes from animals

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**Fig. 1.** Proposed model: Id-specific B cells are stimulated by αCD19-Id targeted to CD19 on B cells in lymphoid follicles.

**Fig. 2.** Design and characterization of αCD19-Id. (A) Design of expression plasmids and schematic of one of four αCD19-Ids. Coexpression of both plasmids in the same CFPS reaction produces two polypeptides that assemble into a noncovalent heterodimeric Db. Locations of heavy chain variable domains (α19 VH and α38 VH), and light chain variable domains (α19 VL and α38 VL) of anti-CD19 and 38C13, respectively, T7 promoters (T7), ribosomal binding sites (rbs), (Gly)4Ser linkers (L), hexahistidine tag (H6), and stop codons (stop) on the expression plasmids are indicated, as are the 38C13 Id and the binding site for CD19 on the Db. (B) Flow cytometry analysis of αCD19-Id bispecific binding. A20 cells were incubated with CFPS products containing 5 μg of each αCD19-Id variant (−) or with mock CFPS product (shaded). A20/CD19<sup>−/−</sup> cells incubated with the same CFPS product (−−) served as a negative target cell control. Cells were then washed and stained with Alexa Fluor 488-conjugated anti-38C13 mAb.
vaccinated with RatFv-Id and αCD19-Id proliferated (Fig. S7) and secreted gamma IFN (IFN-γ) (Fig. 6B) to both the specific and nonspecific Db. There was no response to anti-CD19 mAb or to rat IgG (Fig. 6B). These results indicate that it was a component other than the Ig variable regions (i.e., the linker) shared by both Db, that provided T-cell responses and help to Id-specific B cells. Indeed, peptides most likely to bind the major histocompatibility complex II (MHCII) expressed by C57BL/6 mice (http://imed.med.ucm.es/Tools/rankpep.html) fall within the V-domain linker junction (FDYWQGQTTLTVSSGGGSDIVMTQS) shared by both Db. It is suprising that animals immunized with the vaccine containing a complex with anti-CD19 and avidin did not have activated T cells specific for these xenogenic Ags. However, the lack of such helper T cells may explain the poor anti-Id antibody response induced by this complex.

**Vaccination with αCD19-Id Protected Mice from a Systemic Tumor Challenge.** Vaccinated mice were challenged with lethal doses of the aggressive 38C13 lymphoma. Mice vaccinated with RatFv-Id showed no protection compared with unvaccinated mice. In contrast, mice that received αCD19-Id were protected to a similar degree as those vaccinated with 38C13-KLH (Fig. 7). Tumors from animals vaccinated with αCD19-Id or 38C13-KLH still bound to immune sera generated by these vaccines. Therefore, the lack of protection for these animals could not be explained by the expansion of tumor cells expressing Id variants.

**Discussion**

Patients with follicular lymphoma can be induced to make anti-Id antibodies against their tumors. Those who make such a response have improved overall survival compared with those who do not (2, 3). However, randomized controlled trials have failed to prove a clinical benefit from Id vaccination (5–7). An explanation for this discrepancy may be that the ability to make anti-Id antibody is simply an indicator for which the patient is destined to survive longer. An alternative explanation is that anti-Id antibodies are protective against tumor growth, but only if the response is robust. In one phase III trial, all of the patients produced antibodies against the KLH carrier protein, indicating a certain level of general immune competence, but more than half of them failed to generate anti-Id antibodies (5). One possible problem may have been the chemical conjugation to KLH, a process that is difficult to control, especially by the glutaraldehyde method that was used. It has been established that glutaraldehyde can damage antigenic determinants of an Id and abrogate tumor protection in that animal model (8). For patients
where each Id is unique, the conjugation chemistry may affect each product to a different degree. Therefore, new vaccines that do not require chemical conjugation may lead to improved immunogenicity and clinical outcomes. To achieve this goal, we and others have tested various forms of recombinant Id vaccines (17, 19–23). A common approach is to produce fusions of Id sequences to targeting moieties that direct the construct to cytokine receptors or to other activating receptors on dendritic cells, macrophages, and other antigen-presenting cells (APCs) (19, 22, 23). The peptides derived from Id proteins would then be presented to T cells (24, 25).

Herein, we report an alternative strategy designed to activate Id-specific B cells. This approach targets Id to the surface of noncognate B cells where they can be presented as intact molecules to cognate B cells. Vaccines targeted to the complement receptor 2 (CD21) expressed on a variety of immune cells, including B cells, have been constructed by several groups. Some showed enhancement of Ag-specific immunity (26, 27), whereas others reported unexpected suppression of antibody responses (28, 29). We chose to target Id to the CD19 molecule expressed exclusively on B cells. There is no competing ligand for CD19 as there is for CD21. Importantly, it is known that the majority of CD19-antibody complexes remain on or recycle to the surface of B cells even after extended periods (30, 31). Furthermore, coligation of CD19 to the BCR lowers the activation threshold of B cells (15).

Syngeneic Ig are poor immunogens. However, Id mixed with complete Freund’s adjuvant can generate anti-Id antibody to protective levels in several tumor models (32, 33). Interestingly, no anti-Id antibody can be induced this way in the 38C13 model (34). The unusually poor immunogenicity of this Id may be due to the lack of somatic mutation in its V<sub>H</sub> and V<sub>L</sub> genes (35), resulting in a paucity of CD4<sup>+</sup> T-cell epitopes. The 38C13 Id can be made immunogenic by coupling it to KLH. KLH binds to natural antibodies and complement (36), and has been shown to be transported into the follicles of lymph nodes (14). KLH also contains peptide epitopes that activate CD4<sup>+</sup> T cells. These properties make KLH an effective carrier.

We show that a robust anti-Id antibody response can also be induced by fusing 38C13 Id to anti-CD19. Although different from Id-KLH, αCD19-Id may achieve similar immune-stimulatory functions by alternative strategies. Being small, Db can enter follicles through conduits, as inferred from the speed that αCD19-Id reached B cells in the lymph node (Fig. S3A). Similar conduit systems have been found to channel small molecules into the T-cell areas of a lymph node, and into the white pulp of the spleen (37). αCD19-Id may also be actively transported into spleen follicles by marginal zone B cells expressing CD19. αCD19-Id anchored to CD19 on abundant noncognate B cells provided cross-linking of BCRs on the cognate B cell. Indeed, αCD19-Id bound to B cells induced a stronger BCR signal than free αCD19-Id, and reached the level induced by the pentameric 38C13 IgM (Fig. 4 A and B and Fig. S5). CD4<sup>+</sup> T cells were required for the anti-Id response generated by αCD19-Id. The rat variable regions of anti-CD19 might have been expected to be the source of CD4<sup>+</sup> T-cell epitopes. However, instead, our data indicate that the nonnatural Gly<sub>3</sub>Ser linker provided such epitopes (Fig. 6B and Fig. S7).

The potential to generate immune-stimulatory epitopes is another advantage of recombinant Id vaccines over native Ig Id vaccines, in addition to avoiding the regulatory T-cell epitopes found on Ig constant regions (38). Ding et al. reported that B cells targeted by an anti-CD19-Ag conjugate could prime CD4<sup>+</sup> T cells (39). We have no evidence for this because the nontargeting RatFv-Id was as effective as αCD19-Id in activating T cells. It is likely that some molecules of both Dbs were internalized and presented to T cells by macrophages or dendritic cells. However, in addition, some αCD19-Id targeted to noncognate B cells where they formed an array to present the Id to cognate B cells. By contrast, the nontargeting RatFv-Id induced no anti-Id antibody response, nor did the 38C13 IgM, a good cross-linker of Id-specific BCR but lacking T-cell epitopes. Together, these results underscore the importance of vaccines such as αCD19-Id that are designed to activate both cognate B cells and CD4<sup>+</sup> T cells.

Rituximab is now a part of the standard therapy for follicular lymphoma, therefore, therapeutic vaccine strategies for lymphoma will need to be used in conjunction with this mAb that depletes normal B cells. Rituximab can blunt antibody responses to new Ags but it does not ablate an existing response once it is established by prior vaccination (40, 41). Id vaccines produced rapidly by cell-free protein synthesis, as tested here, can be available before rituximab is used. This strategy may have the additional benefit of delaying the use of rituximab, and therefore, the development of rituximab resistance.

**Materials and Methods**

**Plasmids.** To construct expression plasmids for Dbs, RNAs were extracted from hybridomas producing the anti-CD19 rat IgG2a/s (1D23) (18) and a rat IgG2a/x of irrelevant specificity (H22-15-S) (RNeasy; Qiagen). The V<sub>H</sub> and V<sub>L</sub> genes were isolated using the SMART RACE kit (Clontech) and primers specific to rat IgG2a constant region 1 (5′-gaaatagcccttgaccaggcatc-3′) and
Fig. 6. Immune responses to αCD19-Ig. (A) αCD19-Ig induced a robust Id-specific antibody response. Mice received four biweekly i.d. vaccinations given twice on consecutive days. Vaccines consisted either of 6 µg Dbs or an Id molar equivalent of 38C13 IgM, either chemically conjugated to KLH (38C13-KLH), mixed with anti-CD19 mAb (αCD19 + 38C13), or conjugated to anti-CD19 mAb by avidin (αCD19-Av-38C13). Sera collected a week after the last immunization were tested by ELISA for antibodies against the 38C13 Id. Data were combined from four studies. The number of animals in each group is indicated. Each bar on the graph represents the mean serum anti-Id IgG concentration ± SEM of each group. None of the sera reacted with a mouse IgM isotype control. (B) IFN-γ production by splenocytes from immunized mice. Mice (two to three per group) were vaccinated as in A. Spleens from each group were harvested and pooled a week later. Splenocytes were cultured for 4 d with Ags listed in the legend in hexaplicate wells each. IFN-γ production by splenocytes from immunized mice. Mice (two to three per group) were vaccinated as in A. Spleens from each group were harvested and pooled a week later. Splenocytes were cultured for 4 d with Ags listed in the legend in hexaplicate wells each. IFN-γ production by splenocytes from immunized mice.

Fig. 7. Vaccination with αCD19-Ig protected mice from tumor. (A) Mice (10 per group) received αCD19-Ig, RatFv-Ig, 38C13-KLH, or buffer as described in Fig. 6A. Ten days later, mice were challenged with 100 38C13 cells by i.v. injection. (B) Mice (10 per group) were vaccinated with αCD19-Ig, 38C13-KLH, or buffer and challenged with 400 cells. Survival was analyzed by the Kaplan–Meier method and the log-rank statistical test.

An analysis of potential secondary structures in the upstream 58 nucleotides and the codons of the first nine amino acids was performed using the online resource, Mfold. Silent codon changes were made to eliminate G–C pairings that stabilize secondary structures, which may impede translation. Overlapping oligonucleotides of the coding regions were designed (DNAworks), purchased (IDT), assembled by PCR, and cloned into pY71. The plasmid expressing a membrane-bound anti-38C13 IgM, created for the present work, has been described (42).

Flow Cytometry. Db in vitro binding assay. Cells (10^6) were incubated with CFPS products for 1 h on ice, then with 1 µg Alexa Fluor 488-conjugated 51C5 for 30 min. Cells were washed after each incubation, fixed with 2% (wt/vol) paraformaldehyde, and analyzed on a FACScalibur (Becton Dickinson). Db in vivo trafficking studies. Mice were injected i.d. on the abdomen or i.v. in the tail with 10 µg Alexa Fluor 488 conjugates. Cells isolated from the indicated body compartments were incubated with Fc blocker, stained with fluorophore-conjugated mAbs, washed, fixed, and analyzed as described above. The animal study protocol was approved by the Stanford University Institutional Animal Care and Use Committee.

Db transfer studies. A20 or A20/xBSCR cells at 10^6 cells/mL in PBS were incubated with 5 µM CellTrace Violet dye (Invitrogen) for 20 min at 37 °C, washed, and cultured overnight in full media before use. Splenocytes were harvested from mice injected i.v. with buffer or 20 µg Alexa Fluor-488 conjugated αCD19-Ig. A total of 3 × 10^5 spleen B cells were mixed with 3 × 10^5 Violet dye-labeled cells, centrifuged for 30 s, and incubated for 1 h at 37 °C. Cells were washed with cold PBS, fixed for 30 min in BD CytoFix/CytoPerm solution, washed with BD Perm/Wash buffer, and incubated for 30 min with PE-conjugated mAbs. After washes, cells were fixed and analyzed.

ELISAs. Serum anti-Id IgGs were quantified as described previously (17). To quantify IFN-γ, splenocytes were seeded (5 × 10^4 cells per well) in 96-well U-bottom plates in 100 µL media (5% FBS, 100 µg/mL gentamycin). A final concentration of 50 µg/mL of 38C13 IgM, anti-CD19, respective isotype control antibodies, 10 µg/mL of KLH, avidin, or 2 µg/mL of Dbs was added. Culture supernatants were tested with an IFN-γ ELISA kit (Thermo Scientific).

ACKNOWLEDGMENTS. We thank D. Czerwinski and R. Rajapaksa for technical expertise in flow cytometry; A. Virrueta for technical assistance; C.-C. Kuo for advice on molecular biology; and R. Houot and H. Kohrt for producing the anti-CD4 mAb. This work was supported by a Leukemia and Cancer Institute grant R01 CA 115155 (to I.D.) and the Leukemia and Lymphoma Society and the NCI Cancer Center Support Grant P30 CA 008278.
Lymphoma Society Specialized Center of Research (SCOR) program grant, Ruth L. Kirshstein Grant 5 T32 AI07290 (to P.P.N.), and a Lymphoma Research Foundation fellowship (to P.P.N.). R.L. is an American Cancer Society clinical research professor.


