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Immunology

Epitope-specific tolerance induction with an engineered immunoglobulin

(protein engineering/immune self-tolerance/gene therapy/antigen presentation)

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ABSTRACT **Isologous and heterologous immunoglobulins have been shown to be extremely effective as tolerogenic carriers for nearly 30 years. The efficacy of these proteins is due in part to their long half-life *in vivo*, as well as their ability to crosslink surface IgM with Fc receptors. The concept of using IgG as a carrier molecule to induce unresponsiveness in the adult immune system has been exploited for simple haptens, such as nucleosides, as well as for peptides. To further evaluate the *in vivo* potential of these molecules for inducing tolerance to a defined epitope, we have engineered a fusion protein of mouse IgG1 with the immunodominant epitope 12–26 from bacteriophage λ cI repressor protein. This 15-mer, which contains both a B-cell and T-cell epitope, has been fused in-frame to the N terminus of a mouse heavy chain IgG1 construct, thus creating a "genetic hapten-carrier" system. We describe a novel *in vitro* and *in vivo* experimental system for studying the feasibility of engineered tolerogens, consisting of a recombinant flagellin challenge antigen and a murine IgG1 tolerogen, both expressing the λ repressor epitope 12–26. Herein, we show that peptide-grafted IgG molecules injected i.v., or expressed by transfected, autologous B cells, can efficiently modulate the cellular and humoral immune responses to immunodominant epitopes. This model displays the feasibility of "tailor-designing" immune responses to whole antigens by selecting epitopes for either tolerance or immunity.**

The immune system normally begins to develop tolerance to self-constituents during early development (1). Evidence exists for multiple mechanisms that maintain self-tolerance in B and T cells, including clonal deletion, clonal anergy, and idiotypic suppression (2–4). Self-reactive T cells as well as autoantibodies have been implicated in a variety of autoimmune syndromes, and various models of autoimmunity have shown that tolerance to certain autoantigens is incomplete, entirely absent, or can be disrupted by external factors. In most cases, inadequate presentation to the immune system plays a role (especially for cryptic/subdominant self-epitopes) (5). The specific suppression of undesirable immune responses in immunocompetent individuals is an ultimate clinical goal, and induction of tolerance to putative autoantigens as well as immunodominant T-cell epitopes of allergens has already been proposed (6–8).

Experimental tolerance induction of the adult immune system has been described extensively for diverse haptens, including nucleosides and peptides (9–17). Chemical coupling of epitopes to IgG carriers, however, can be limited by the availability of free reactive amino groups, structural change of the epitope as a result of the coupling reaction, and the uncontrolled targeting of the added determinant to different portions of the IgG.

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Protein engineering strategies have been used to create molecules containing heterologous epitopes for the amplification of specific immune responses. For example, heterologous oligopeptide epitopes of immunological interest have been inserted in-frame into bacterial flagellin (18), influenza virus nucleoprotein (19), and the complementarity-determining regions (CDR) of immunoglobulins (20–22). Attempts have been made to test the ability of such recombinant proteins to specifically generate enhanced immune responses to the heterologous oligopeptide. However, the induction and maintenance of tolerance to oligopeptides presented to the immune system by engineered proteins have not yet been demonstrated. Furthermore, essential to any tolerization protocol is a method of maintaining a specific state of tolerance. Such maintenance of tolerance normally requires the persistence of the epitope *in vivo* (23–25).

In this study, we have taken advantage of the IgG molecule as a tolerogenic carrier and have created an engineered tolerogen with a grafted epitope at the N terminus of an IgG H chain. This engineered IgG is shown to be recognized by the immune system in a tolerogenic manner. The model epitope we have chosen is the well-characterized class II major histocompatability complex-restricted peptide sequence from the λ cI repressor protein (p1–102), residues 12–26. This peptide contains both a B- and T-cell epitope and is the immunodominant determinant in H-2^d mice immunized with the entire protein (26–30). We are thus able to measure tolerance induction to a single determinant at both the B-cell and T-cell levels in this model. Furthermore, tolerogenic Ig-peptide constructs have the potential to be expressed in adoptively transferred hematopoietic tissue for the permanent modulation of epitope-specific immune responses in mature adults. Such an approach may provide new insights on the nature of peripheral immune tolerance, and it complements transgenic studies in which neo self-antigens are expressed in the context of a developing immune system (2, 3). Our experiments show that 12–26-IgG is an efficient tolerogen in adult mice and can provide a model system for future epitope-specific tolerance induction and maintenance strategies.

MATERIALS AND METHODS

Mice. Male and female BALB/cByJ (H-2^d) and CAF1 (H-$2^{d/a}$) mice were obtained from The Jackson Laboratory and were used at 6–10 weeks of age.

Reagents and Immunologic Methods. *Medium.* RPMI 1640 medium (GIBCO/BRL) was supplemented with 5% fetal calf serum (HyClone), 2-mercaptoethanol, L-glutamine, penicillin,

Abbreviations: AP, alkaline phosphatase; APC, antigen-presenting cell; CFA, complete Freund's adjuvant; LN, lymph node; Th, T-helper cell subset; HEL, hen egg lysozyme; IL, interleukin; FR1, first framework region; PPD, purified protein derivative.

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streptomycin, MEM nonessential amino acids, and sodium pyruvate.

Antibodies. Hybridoma B3.11, which produces a monoclonal IgG1 specific for the 12–26 peptide, was a kind gift of Tom Briner and Malcolm Gefter (Immulogic, Waltham, MA). B3.11 was affinity purified with goat anti-mouse IgG Sepharose columns and biotinylated, or used as a neat culture supernatant. All alkaline phosphatase (AP)-conjugated reagents were purchased from Southern Biotechnology Associates.

Synthetic peptide. The 12–26 15-mer LEDARRLKAIYEKKK was prepared with a solid-phase method and purified to >92% homogeneity using standard HPLC methods. Peptide was conjugated to BSA, rabbit gamma globulin, or keyhole limpet hemocyanin as described (28).

Oligonucleotides. The following complementary synthetic oligonucleotides encoding the 12–26 sequence were designed with *Bam*HI/*Cla*I restriction ends, phosphorylated with T4 kinase and ATP, and cloned into the hypervariable region of flagellin construct pPX1647: DWS1, 5′-CGA TCT GGA GGA CGC GCG GCG GCT GAA GGC GAT ATA CGA GAA GAA GAA GG-3′; DWS2, 5′-GAT CCC TTC TTC TTC TCG TAT ATC GCC TTC AGC CGC CGC GCG TCC TCC AGA T-3′.

PCR primers were also designed to amplify a modified 12–26 sequence from the chimeric 12–26-flagellin construct. This sequence includes the 5′ first framework region (FR1) V_H sequence and *Pst*I restriction sites at each flanking end: Ig-one, 5′-TGA TCT ACT GCA GCT GGA GGA CGC GCG GCG G-3′; Ig-two, 5′- CGA CCT CCT GCA GTT GGA CCT GCT TCT TCT TCT CGT ATA T-3′.

ELISA. To determine the specificity of binding of our peptide-specific mAb B3.11 to 12–26 fusion proteins, competitive inhibition ELISAs were conducted as follows. Biotinylated B3.11 was incubated 1:1 (vol/vol) with decreasing amounts of inhibitor in ELISA binding buffer (0.25% BSA/0.05% Tween 20 in saline). Mixtures were then incubated on peptide-coated (10 μg/ml) ELISA plates (Immulon 4, Dynatech), and subsequently, streptavidin-AP was added as a secondary reagent. Percent inhibition of binding (A_{405}) was calculated as: [(average binding of antibody alone − average binding of antibody incubated in presence of inhibitor)/average binding of antibody alone] × 100. ELISA determinations of serum peptide-specific IgG responses were done by coating ELISA plates with 50 μg/ml of synthetic peptide. Antigen-coated plates were blocked with 1% gelatin/0.05% Tween 20 buffer, and duplicate serial dilutions of serum were incubated and probed with goat anti-mouse IgG isotype-specific secondary reagents. Titers are expressed as the geometric mean of the reciprocal dilution required to bring A_{490} readings to prebleed levels or <0.08.

Protein Engineering Design. *Preparation of a recombinant* Salmonella flagellin *construct encoding the 12–26 peptide sequence in a hypervariable region.* Plasmid pPX1647 containing the 1530-bp H1-d flagellin gene (originally cloned in pBR322) from *Salmonella münchen* (ATCC 8388) was a kind gift of Robert Brey (Praxis, Rochester, NY). Complementary oligonucleotides coding for the λ cI repressor 12–26 sequence were cloned into pPX1647 at a *Bam*HI/*Cla*I polylinker region, and a 12–26-flagellin recombinant plasmid (pA29) and the wild-type flagellin construct (pPX) were electroporated into SL5927 (Δ*fla*, *aroA*), a flagellin-deficient *Salmonella dublin* mutant as described (18). Polymeric flagellins were prepared and purified essentially as described earlier (31).

Preparation of a murine H chain IgG1 construct encoding the 12–26 sequence at the N terminus. Our strategy for inserting a foreign peptide sequence at the N terminus of an IgG H chain is similar to what has been described (32, 33). Plasmid pSNR (34), which contains neomycin and ampicillin resistance genes, as well as the full genomic sequence for a IgG1b H chain cross-reactive with the 5-iodo-4-hydroxy-3-nitrophenylacetyl (NIP) hapten, was obtained from Douglas Fearon (Cambridge University) and modified. Briefly, a modified 12–26 sequence was created by PCR amplification of this sequence from our chimeric flagellin construct A29 (described above) using PCR primers Ig-one and Ig-two. The modified 12–26 sequence was subcloned into the V_H site of pSNR, and recombinant clones were analyzed for proper orientation and *Taq* DNA polymerase mutational errors by double-stranded DNA sequencing methods (Sequenase 2.0 kit, United States Biochemical).

Expression, purification, and quantitation of transfected IgG. Construct pQ3.EZ1 (Q3), as well as the control pSNR IgG1 construct (P6) were electroporated into J558L myeloma cells (which produce only a λ L chain) as described (32–34). Stably transfected clones were isolated in 1 mg/ml of G418 (GIBCO/BRL) and subcloned, and transfected IgGs from selected clones were purified from bulk supernatants or ascites fluid with anti-mouse IgG-Sepharose or protein G columns. Because the original H chain binds with high affinity to the NIP hapten, purified or serum transfectoma IgG was quantitated using a modified NIP-gelatin binding ELISA (32, 33), with the use of anti-mouse IgG1-AP as a secondary reagent.

***In Vitro* and *in Vivo* Tolerance Induction and Immunization Protocols.** Peptide-specific tolerance induction in adult recipients was accomplished by i.v. injection (lateral tail vein) of either 1 mg of purified, deaggregated, chimeric (Q3) or control IgG (P6) diluted in saline, or by three repeated injections of mitomycin C-treated (50 μg/ml; Sigma) P6- or Q3-secreting transfectomas. For measurement of humoral immune responses 10 days after i.v. tolerization, animals were immunized s.c. at the base of the tail and i.p. with 50 μg of synthetic 12–26 peptide and 25 μg of hen egg lysozyme (HEL; Sigma) emulsified in complete Freund's adjuvant (CFA; Sigma). Mice received an additional antigenic boost of 50 μg of peptide and 25 μg of HEL injected i.p. in saline 2 weeks after initial priming. Mice were bled for serum anti-peptide antibody responses 8 days after this boost. Splenic memory T-cell (tertiary) responses were analyzed in culture 8 weeks after secondary boosts. Splenic T cells were enriched by panning on anti-Ig-coated plates and were restimulated (3×10^6 cells per ml) with dilutions of peptide and irradiated splenic antigen-presenting cells [APC; 2500 rads (1 rad = 0.01 Gy), 1×10^6 cells per ml). For analysis of secondary lymph node responses after i.v. tolerization, animals were immunized in the hind footpads with 20 μg of peptide emulsified in CFA, and draining popliteal lymph nodes were harvested 9 days later and restimulated with dilutions of peptide and 50 μg/ml of purified protein derivative (PPD; Connaught Laboratories). Interleukin 2 (IL-2) and IL-4 were determined from cultured supernatants at 24 and 48 hr, respectively, in lymph node or splenic T-cell cultures using recombinant cytokines as standards (27, 35).

In vitro B-cell tolerance induction experiments were done on enriched splenic B cells essentially as described before (15–17). Supernatants from 3- to 4-day cultures were assayed for IgM production by ELISA by coating wells with peptide- or fluorescein isothiocyanate-BSA conjugates, and probing with goat anti-mouse IgM-AP. Data are representative of experiments repeated two to three times; individual points represent the arithmetic mean of triplicate/quadruplicate values with standard deviations generally less than 15% (omitted from the figures for clarity).

RESULTS

Preparation and Characterization of a Murine IgG1 Self-Carrier Containing the λ cI Repressor 12–26 Peptide at the V_H N Terminus. The 12–26-IgG construct was prepared by modifying plasmid pSNR (34), which contains the genomic sequence encoding a murine IgG1b H chain. Isologous IgG1 was

chosen because of its documented activity as a tolerogenic "carrier" that is equal to that of IgG2 and greater than other immunoglobulin isotypes or serum proteins (15). We chose to insert a foreign epitope at the N terminus of the H chain variable region (Fig. 1*A*), because such insertions have been shown not to alter normal immunoglobulin folding and structure (32, 33). Analysis of transfected, purified chimeric 12–26-IgG (Q3) or control pSNR IgG (P6) by SDS/PAGE (Fig. 1*B*) shows that H chains can successfully pair with J558L L chains. This Coomassie stain also shows that the chimeric H chain (Q3) containing the additional 12–26 sequence is ≈1.8 kDa larger than the control IgG (P6).

Purified transfected IgGs were shown to express the 12–26 epitope by Western blotting and ELISA using peptide-specific mAb B3.11 (Fig. 1*C*). Furthermore, competitive inhibition ELISA studies (Fig. 1*D*) show that chimeric 12–26-IgG can effectively compete with free synthetic peptide or a chemical

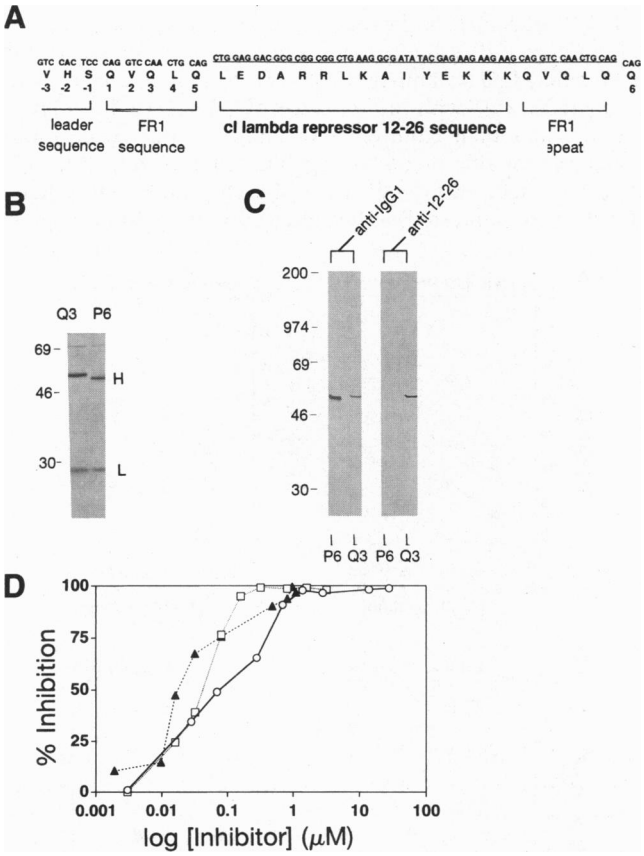

FIG. 1. Strategy for the construction, expression, and epitope recognition of a 12–26-V_H gene. (*A*) A modified 12–26 nucleotide sequence was ligated into a *Pst*I site of a 1.3-kb murine V_H (LVDJ) chain fragment as described in the text. The *Pst*I site appears at the coding sequence of the fifth amino acid of the FR1; therefore, a repeat of the first five FR1 amino acids was designed to follow the coding sequence of the 15 amino acids of 12–26, so as not to perturb proper framework region folding after insertion. (*B*) Coomassie stain of a SDS/10% polyacrylamide gel of purified H-chain transfected immunoglobulins shows proper assembly of H chains with L chains. (*C*) Recognition of epitopes by immunoblotting. Purified control IgG (P6) or 12–26-IgG (Q3) samples were electrophoresed on SDS/10% polyacrylamide gels, transferred onto nitrocellulose, and probed with anti-mouse IgG1 (left lanes) or with biotinylated anti-12–26 mAb B3.11 (right lanes) plus AP-conjugated secondary reagents. (*D*) ELISA inhibition curves. ELISA plates were coated with 10 μg/ml of synthetic 12–26 peptide, and pre-titrated mAb B3.11 was mixed with increasing amounts of the following competitive inhibitors: ○, synthetic 12–26 peptide; □, 12–26 chemically coupled to rabbit gamma globulin; or ▲, Q3. Experimental details are described in the text.

conjugate of 12–26-rabbit IgG for binding to mAb B3.11. These data suggest that the inserted peptide can be recognized efficiently by epitope-specific antibodies/B cells on the exterior surface of the recombinant IgG, without significantly perturbing H chain tertiary structure.

Additionally, the recombinant 12–26-IgG chimera is immunogenic and capable of priming 12–26-specific T and B cells *in vivo*. Mice immunized with Q3 emulsified in CFA were able to prime 12–26-specific T cells comparable to the response elicited with synthetic peptide. *In vitro* restimulation of lymph node (LN) cultures with synthetic peptide resulted in T-cell proliferation (Fig. 2), as well as IL-2 and IL-4 production (data not shown), in peptide- and Q3-primed LN cells but not in P6-primed LN cells. Immunization also leads to a high serum anti-12–26 IgG antibody titer detectable by peptide-specific ELISA (data not shown). Additional data (unpublished data) shows that 12–26-IgG can stimulate IL-2 production in an I-A^d-restricted 12–26-specific T-cell hybridoma (9C127) (27). These results suggest not only that the inserted epitope's conformation is recognized by specific antibodies, but also that the peptide (or one extremely similar to it) can be processed and presented to T cells in a physiologically relevant manner by APC, even in the context of a self-IgG scaffold.

***In Vivo* Induction of Peptide-Specific Immune Self-Tolerance with Soluble Purified Engineered 12–26-IgG.** To test the efficacy of genetically engineered tolerogens, we analyzed both humoral and cellular responsiveness after i.v. administration of high doses of soluble, deaggregated 12–26-IgG. Mice were injected with 1 mg of either chimeric Q3 or control P6 immunoglobulin and challenged 10 days later with a mixture of 12–26 peptide and HEL (as a specificity control) emulsified 1:1 in CFA. Secondary humoral immune responses were analyzed 1 week after an additional boost. Fig. 3*A* shows that mice receiving pretreatments of Q3, but not control P6, are dramatically unresponsive to peptide challenge as assessed by anti-peptide IgG ELISA measurements, whereas control anti-HEL titers were unaffected. Although the main anti-peptide isotype in BALB/c mice is IgG1, all isotypes, including

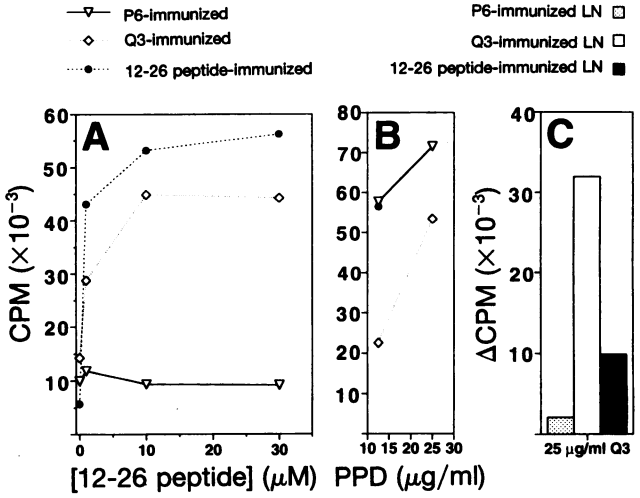

FIG. 2. *In vivo* priming of 12–26-specific T cells by 12–26-IgG fusion protein. The proliferative response of T-cell-enriched LN cells from CAF1 mice immunized with 100 μg (55.6 nmol) of synthetic 12–26 peptide, 100 μg (0.67 nmol) of control IgG (P6), or 100 μg (0.65 nmol) of 12–26-IgG1 fusion protein (Q3) was determined 9 days later. Responses to 12–26 peptide (*A*), PPD (*B*), or whole purified Q3 (*C*) were assessed by [^{3}H]thymidine incorporation on day 4 of culture. Cultures were set up in triplicate from pooled LN cells of three individually immunized mice. ▽, P6-immunized; ◇, Q3-immunized; ●, 12–26 peptide-immunized; stippled bar, P6-immunized LN; open bar, Q3-immunized LN; filled bar, 12–26 peptide-immunized LN.

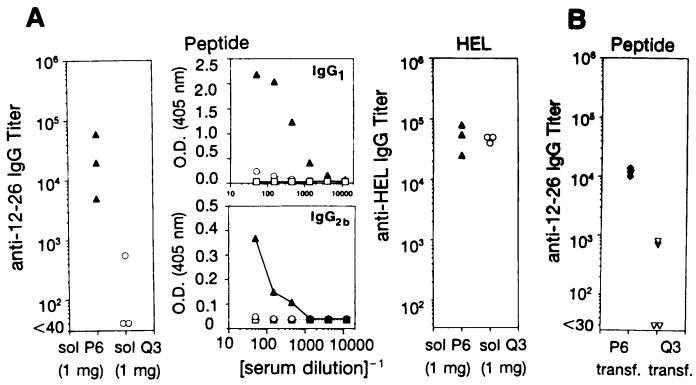

FIG. 3. *In vivo* effect of 12–26-IgG pretreatment on peptide-specific humoral immune responses. Male BALB/c mice were injected i.v. with either (*A*) a single 1-mg dose of deaggregated protein G-purified P6 (▲) or Q3 (○) IgG or (*B*) three weekly injections of 5×10^6 syngeneic IgG-secreting transfectomas P6 (◆) or Q3 (▽), which had been pretreated with mitomycin C. Mice were immunized as described in text, and total or isotypic HEL or peptide-specific IgG titers were quantitated by ELISA 8 days after a secondary antigenic boost. Peptide-specific isotype titrations (IgG1 and IgG2b) represent mean ELISA values from three individual mice in each group. □, Preimmunization serum dilutions.

IgG2b, were consistently diminished by 12–26-IgG pretreatment (Fig. 3*A*).

To test the potential of inducing unresponsiveness with peptide-Ig-transfected cells as a model for gene-therapy tolerogenesis, we injected BALB/c mice with three consecutive weekly i.v. injections of transfectomas secreting Q3 or P6 control IgG that had been pretreated with mitomycin C. Such treatment results in transient serum expression of transfected IgGs of at least 10–500 ng/ml (as assessed by NIP-gelatin ELISA). This type of pre-treatment also resulted in diminution of peptide-specific humoral immune responses (Fig. 3*B*), as well as reduction of cellular LN proliferative responses (data not shown).

Because serum antibody unresponsiveness may be a function of either B-cell or T-cell tolerance (or both), the cellular basis of this tolerance was analyzed by measuring T-helper (Th) cytokine responses in these mice 8 weeks after immunogenic challenge. Restimulation of memory splenic T cells (Fig. 4*A*) revealed that both Th1-type (IL-2) and Th2-type (IL-4) responses were absent in tolerized mice, a result consistent with the observed lack of anti-peptide IgG2b and IgG1 (Fig. 3*A*), which depend on these Th subsets. The T-cell response to peptide was further confirmed to be diminished in 12–26-IgG pretreated animals in short-term LN restimulation experiments. Mice tolerized with 1 mg of 12–26-IgG 10 days before peptide challenge had reduced LN IL-2 responses but unaffected PPD recall proliferative responses compared with control P6-injected animals (Fig. 4*B*). These results are consistent with an efficient induction of Th tolerance to the immunodominant peptide, which results in an inability to prime any subset of T-cell response to the peptide.

Thus, foreign immunogenic peptides genetically engrafted into Ig scaffolds can be very efficiently presented to the immune system in a tolerogenic manner when administered by the appropriate route and method. These results show that pretreatment with peptide-Ig chimeras delivered either as single high doses or via slow release by transfected autologous B cells have promising potential to specifically manipulate undesirable T-cell responses in a very efficient manner.

Analysis of a Novel 12–26-Flagellin Immunogen for Testing the Efficacy of 12–26-IgG on B-Cell Tolerance. To test the efficacy of chimeric 12–26-IgG as a B-cell tolerogen, we needed to challenge B cells with an immunogenic T-independent form of the 12–26 epitope. Because polymerized flagellin is a well-characterized T-independent antigen (31), we constructed a 12–26 flagellin fusion protein with a strategy previously described (18). Western blotting and ELISA analyses of purified wild-type (pPX) and 12–26-flagellin (A29) showed that although flagellar epitopes are readily expressed in both flagellins, the inserted epitope was detectable only in chimeric flagellin A29 (Fig. 5*A*). Polymerized 12–26-flagellin was found to stimulate splenic B cells to secrete anti-12–26 IgM comparable with bacterial lipopolysaccharide, a polyclonal B-cell mitogen. A concentration of 0.1 μg/ml was determined to be minimally mitogenic (as assessed by anti-fluorescein IgM ELISAs) and used for subsequent experiments. These data show that our inserted epitope can also be recognized in the context of a polymerized flagellin molecule and can readily stimulate B cells to produce epitope-specific IgM.

In subsequent experiments, we tested the ability of 12–26-IgG to induce B-cell unresponsiveness, as has been shown for haptenated IgG or anti-IgM (15–17). Enriched B cells were incubated *in vitro* with various doses of Q3 or P6 control IgGs, washed, and then cultured with either mitogenic bacterial lipopolysaccharide or 12–26-flagellin. Alternatively, BALB/c mice were injected i.v. with 1 mg of each protein, and splenic B cells were harvested and challenged *in vitro* 10 days later.

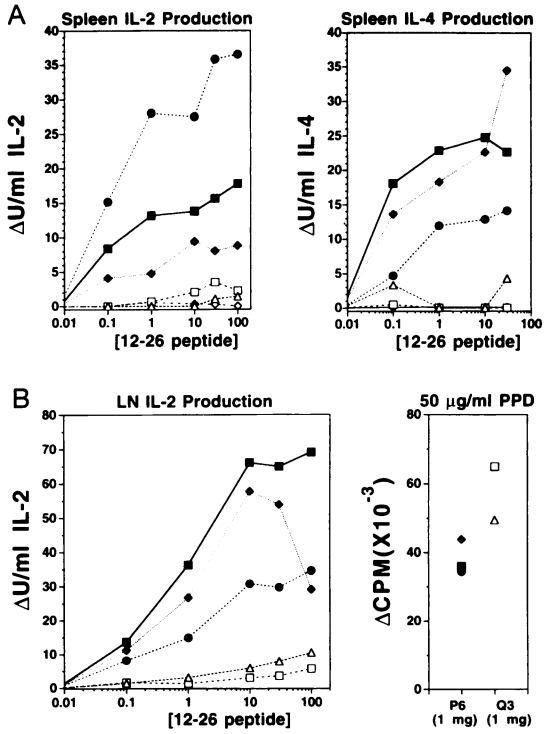

FIG. 4. *In vivo* effect of 12–26-IgG pretreatment on peptide-specific cellular immune responses. (*A*) Tertiary cytokine responses were determined from cultures of enriched splenic T cells (3×10^6 cells per ml) from mice displaying tolerized humoral immune secondary responses (Fig. 3*A*). (*B*) IL-2 production was also assessed for secondary *in vitro* recall responses from cultures of immunized draining LN cells (4×10^6 cells per ml) following i.v. tolerization with 1 mg of either soluble P6 or Q3 IgG. Solid line and filled symbols, individual P6-pretreated mice; dashed lines and open symbols, individual Q3-pretreated mice. PPD proliferative responses serve as an immunization control. IL-2 and IL-4 production in supernatants was determined in triplicate by CTLL and CT.4S assay, respectively, as described (27, 35). Medium-alone backgrounds were subtracted and ranged from 1 to 4 units/ml in all assays.

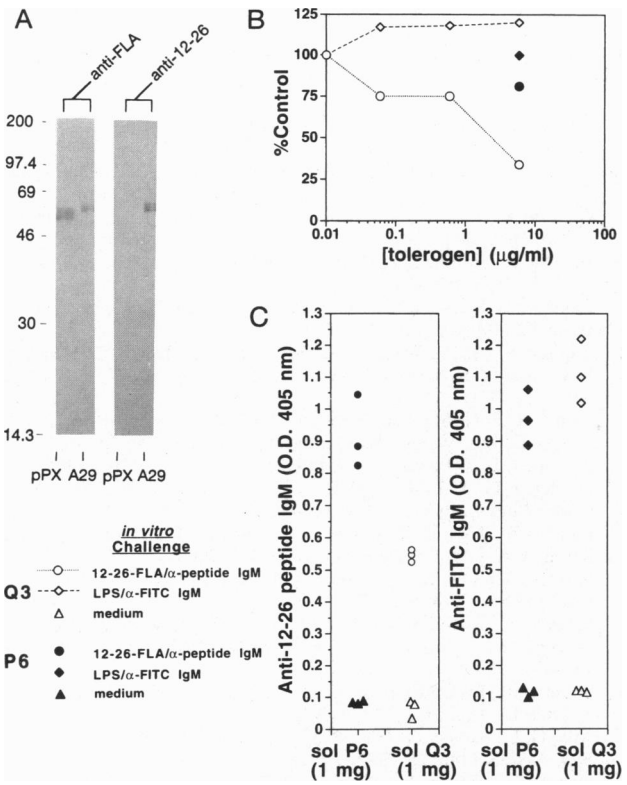

FIG. 5. 12–26-Flagellin fusion protein: a T-independent form of the 12–26 epitope for testing B-cell tolerogenicity of 12–26-IgG. Expression of 12–26 and flagellin epitopes in recombinant 12–26-flagellin was verified by immunoblotting (*A*) and competitive inhibition ELISA (data not shown). Purified wild-type flagellin (pPX) or 12–26-flagellin (A29) was electrophoresed on SDS/10% polyacrylamide gels, and blots were probed with either anti-H1-d flagellin (anti-FLA, left lanes) or anti-12–26 mAb B3.11 (right lanes) (Difco). B-cell tolerance induction analysis was conducted by culturing BALB/c splenic B cells with varying amounts of purified P6 or Q3 *in vitro* (*B*), or by injecting 1 mg i.v. (three BALB/c per group) of each protein *in vivo* (*C*) and analyzing splenic B cells *in vitro* 10 days later. Peptide-specific IgM (α-peptide) ELISA from diluted cultured supernatants was measured after immunogenic challenge of *in vitro*- or *in vivo*-tolerized B cells with 12–26-flagellin. Anti-fluorescein hapten IgM (α-FITC) was measured from tolerized B cells as a specificity control after polyclonal stimulation with 10 μg/ml of bacterial lipopolysaccharide. *In vitro* tolerization responses are presented as percent of control (pretreatment with medium alone).

Supernatants from 3-day cultures were assayed for 12–26-specific IgM or anti-fluorescein hapten as a specificity control. Fig. 5 *B* and *C* shows that pretreatment with Q3, but not P6, either *in vitro* or *in vivo*, results in marked suppression of anti-12–26 IgM, whereas anti-fluorescein control IgM responses were unaffected. These studies show that, in addition to inducing potent Th tolerance, the chimeric IgG is also capable of independently inducing specific unresponsiveness in epitope-specific B cells. B-cell tolerance effects, however, appear to be more modest *in vivo* (Fig. 5*C*) than T-cell effects and may reflect either the requirement for higher epitope multivalency than is provided by a bivalent structure, or higher dosage requirements. A similar magnitude of reduction was also observed for humoral immune responses after adoptively transferring *in vivo*-tolerized B cells plus nontolerized naive T cells into secondary immunodeficient recipients and immunizing with peptide in CFA (unpublished data).

DISCUSSION

The development and maintenance of the unresponsive state in newly emerging lymphocytes is a lifelong process and requires the persistence of antigen (23–25). Exposure of mature B and T cells to antigen in the adult immune system may result in either activation or tolerance, depending on the route and method of exposure, as well as the availability of costimulatory signals from specialized APC (4). Because a primary goal in the treatment of autoimmunity, allergy, and allograft rejection has been to induce specific immune unresponsiveness in adult mature lymphocytes, a variety of approaches have exploited these pathways of exposure for this purpose (36, 37). Of these approaches, experimental tolerance induction with gamma-globulin carriers has been most extensively described. Intravenous administration of soluble, deaggregated IgGs in the absence of adjuvants has been shown to induce antigen-specific B-cell and T-cell tolerance even in the absence of a thymic environment. Mechanisms of specific clonal anergy/inactivation and deletion have been implicated in this type of experimental model (38–42).

In these studies, we describe the tolerogenic capability of an engineered self-IgG expressing a model class II major histocompatability complex-restricted immunodominant peptide: the novel 12–26 epitope from λ cI repressor protein. We have expressed this novel epitope at the N terminus of a murine IgG H-chain construct and have shown it to be tolerogenic *in vivo* and *in vitro*. Conceptually similar approaches have been used to express immunogenic malarial or viral peptides in the CDR3 loop of Ig H chains for the induction of enhanced anti-peptide immune responses (20–22). Zaghouani and coworkers (21) reported that processed peptide-IgG chimeras can efficiently generate a fused immunodominant viral epitope sequence and were superior immunogens compared with free unconjugated peptide when administered with adjuvant. Similarly, our 12–26-IgG protein was highly efficient at inducing peptide-specific cellular and humoral (unpublished data) immune responses when administered in an immunogenic manner (i.e., emulsified in CFA). In the former study (21), it was shown that T-cell activation for a class II-restricted epitope was enhanced 100- to 1000-fold in the context of an Ig-chimera, when presented *in vitro* by stimulatory dendritic APC. Similarly, we observed that approximately a 100-fold lower molar quantity of 12–26-IgG as compared with free peptide was required to stimulate similar numbers of peptide-specific T cells from immunized draining lymph nodes.

The increased efficiency of both immune activation and unresponsiveness induction for Ig-peptide chimeras may follow similar pathways and may be the direct result of the increased half-life, as well as Fc-mediated uptake and presentation of immunoglobulin molecule carriers (43). In the absence of adjuvants (which act by mobilizing APC with efficient costimulatory capabilities), high doses of soluble, deaggregated serum antigens may be preferentially taken up by Fc receptor-mediated endocytosis or phagocytosis and presented by "non-professional" APC, such as resting B cells (44–46). Furthermore, immunoglobulin carriers have the ability to induce efficient B-cell unresponsiveness by mechanisms involving the crosslinking of surface IgM to Fc receptors (15–17). A combination of the mechanisms proposed above may be sufficient in explaining the enhanced efficiency of inducing tolerance with Ig carriers. In contrast, i.v. injection of soluble, deaggregated peptide is sufficient for inducing effective Th tolerance (29) but is incapable of inducing unresponsiveness in peptide-specific B cells (E.T.Z., A. Gaur, and D.W.S., unpublished data). A differential analysis of tolerance induction by peptide-IgG chimeras vs. free peptide may shed light on the nature of requirement for antigenic valency in peripheral B-cell tolerance induction.

Our data indicate that 12–26-IgG fusion protein can present the λ cI repressor epitope in a tolerogenic fashion to induce B- and T-cell tolerance. The uniqueness of this epitope allows us to simultaneously study both cellular and humoral immune responses to a single model immunodominant peptide in a

controlled fashion. The 12–26 peptide induces an extremely vigorous antibody response that has a predominantly IgG1 isotype and can prime Th cells with both a Th1 and a Th2 phenotype (26–30). We found that tolerance induction with 12–26-IgG was globally effective in suppressing every type of immune response that can be elicited by this immunodominant peptide. These results are consistent with previous observations using tolerogenic IgG carriers (11–13, 39, 47–49), but contrast with more recent analyses reporting that, although Th1-type immune responses could be suppressed in a high dose model, tolerance induction was more difficult for Th2-dominated immune responses (50–53). The nature of the conflict between these observations is unclear, but it may reflect either incomplete tolerance induction or perhaps "immune deviation" (54) to the injected antigens, which in some cases included analyses with non-IgG carriers (51, 52).

These studies provide a powerful model to determine the efficacy of inducing specific unresponsiveness to defined antigens or peptides for the modulation of undesirable immune responses. In contrast to H-chain CDR3 insertion of heterologous epitopes, N-terminal addition is not restricted to the size of the antigen fused to the tolerogenic IgG carrier (31, 32). Therefore, both peptides and whole complex foreign antigens may be fused for tolerogenic antigen presentation. Finally, because this system consists of a "genetic hapten-carrier," we propose that these tolerogenic fusion proteins can be used for the induction and long-term maintenance of tolerance if expressed as gene-transferred constructs in hematopoietic tissue. We are currently analyzing the potential of this strategy in transgenic mice expressing engineered 12–26-IgG in the B-cell compartment, as well as in recipients of bone marrow stem cells that have been transduced with a retroviral vector for the long-term expression of peptide-IgG cDNA constructs. Our results thus far have suggested that peripheral B cells expressing peptide-Ig molecules are exceptionally potent in specifically tolerizing mature lymphocytes. Applications of this technology are currently underway for gene-therapy tolerogenesis strategies targeted to known autoantigens (6, 55, 56), inhibitory antibodies that limit hemophilia treatment with recombinant factor VIII or IX (57), and ineffective and potentially harmful antibody responses that occur during HIV infection (58).

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