Rationally Engineered Therapeutic Proteins with Reduced Immunogenicity

Shabnam Tangri, Bianca R. Mothé, Julie Eisenbraun, John Sidney, Scott Southwood, Kristen Briggs, John Zinckgraf, Pamuk Bilsel, Mark Newman, Robert Chesnut, Cynthia LiCalsi and Alessandro Sette

*J Immunol* 2005; 174:3187-3196; 
doi: 10.4049/jimmunol.174.6.3187

http://www.jimmunol.org/content/174/6/3187

---

**References**  
This article cites 41 articles, 15 of which you can access for free at:  
http://www.jimmunol.org/content/174/6/3187.full#ref-list-1

**Subscription**  
Information about subscribing to The Journal of Immunology is online at:  
http://jimmunol.org/subscription

**Permissions**  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
Rationally Engineered Therapeutic Proteins with Reduced Immunogenicity

Shabnam Tangri,* Bianca R. Mothé,*† Julie Eisenbraun,* John Sidney,*‡ Scott Southwood,* Kristen Briggs,* John Zinckgraf,* Pamuk Bilisel,* Mark Newman,* Robert Chesnut,* Cynthia LiCalsi,* and Alessandro Sette†‡

Chronic administration of protein therapeutics may elicit unacceptable immune responses to the specific protein. Our hypothesis is that the immunogenicity of protein drugs can be ascribed to a few immunodominant helper T lymphocyte (HTL) epitopes, and that reducing the MHC binding affinity of these HTL epitopes contained within these proteins can generate drugs with lower immunogenicity. To test this hypothesis, we studied the protein therapeutic erythropoietin (Epo). Two regions within Epo, designated Epo 91–120 and Epo 126–155, contained HTL epitopes that were recognized by individuals with numerous HLA-DR types, a property common to immunodominant HTL epitopes. We then engineered analog epitopes with reduced HLA binding affinity. These analog epitopes were associated with reduced in vitro immunogenicity. Two modified forms of Epo containing these substitutions were shown to be bioactive and nonimmunogenic in vitro. These findings support our hypothesis and demonstrate that immunogenicity of protein drugs can be reduced in a systematic and predictable manner. The Journal of Immunology, 2005, 174: 3187–3196.

Over the last few years, an increasing number of protein drugs have entered clinical trials or received approval for product registration. Several of these drugs are recombinant hormones, lymphokines and growth factors, such as insulin, factor VIII, IFNs, IL-2, and GM-CSF. Abs, such as Remicade (anti-TNF), Rituxan (anti-CD20), and Herceptin (anti-HER2/Neu) also add to this list of registered protein drugs.

These products are commonly administered at high doses over prolonged treatment periods, and a complication associated with their use is the development of protein-specific immune responses. Of particular concern are Abs that may render the drugs less effective; examples include the development of Abs against factor VIII in hemophiliacs (1, 2), calcitonin in patients treated for osteoporosis (3, 4), erythropoietin (Epo)2 in patients undergoing therapy for chronic renal failure (5, 6), and IFN-β in individuals undergoing treatment for multiple sclerosis (7).

Not all protein drugs induce an immune response in a high percentage of patients. However, exceptionally immunogenic products are known to exist, such as IFN-β and factor VIII, where as much as 30–40% of patients develop neutralizing Abs (1, 7). Unwanted immune responses against protein drugs can pose safety risks to patients in addition to simply reducing drug potency. For example, the development of Abs specific to Epo was recently associated with red cell aplasia (5, 6).

The development of Ab responses generally requires activation of helper T lymphocyte (HTL) (8), a process that is dependent on peptide epitopes binding to HLA class II molecules (9, 10). Binding affinity is directly correlated to the ability of the peptide epitopes to elicit immune responses (11). Thus, proteins devoid of HTL epitopes would be expected to be less immunogenic and, therefore, better candidates for use as therapeutic drugs.

Typically, immunization with protein Ags results in immune responses that are not specific for numerous epitopes. Rather, only a small fraction of all possible epitopes induces responses (12–14). Immunodominance is caused, in part, by the fact that only a limited number of all possible epitope peptides bind to individual MHC molecules, a phenomenon termed “determinant selection” (15). Immunodominance is also correlated with binding to multiple HLA class II molecules, a phenomenon referred to as degenerate binding (16, 17). This correlation between highly degenerate HLA-DR binding and immunodominance has been studied in detail from several sources including tetanus toxin, influenza hemagglutinin, hepatitis C virus, hepatitis B virus, and Plasmodium falciparum (18–21). Immunodominant epitopes that are degenerate binders have also been reported for proteins derived from self-Ags, such as myelin basic protein and myelin proteolipid protein (22, 23).

Our working hypothesis is that immunodominant HTL epitopes present in protein drug products can be identified and modified to reduce the immunogenicity of the protein. Specifically, we hypothesize that altering immunodominant HTL epitopes to reduce the HLA-DR binding affinity will reduce their intrinsic immunogenicity. In the present study, we analyzed five protein therapeutics for which evidence of immunogenicity in humans exists (24), namely, calcitonin, Epo, human growth hormone (hGH), IFN-β, and insulin. All five proteins were analyzed for the presence of immunodominant HTL epitopes, and the Epo protein was subsequently chosen to test our hypothesis. Specifically, two regions with Epo which contained immunodominant HTL epitopes were engineered with analog epitopes and tested for immunogenicity in vitro.

*Epimmune, San Diego, CA 92121; †California State University, San Marcos, CA 92096; and ‡La Jolla Institute for Allergy and Immunology, San Diego, CA 92121

Received for publication August 19, 2004. Accepted for publication December 17, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Address correspondence and reprint requests to Dr. Alessandro Sette, 3303 Bunker Hill Street, Suite 326, San Diego, CA 92109. E-mail address: alex@liai.org

2 Abbreviations used in this paper: Epo, erythropoietin; HTL, helper T lymphocyte; SFC, spot-forming cell; hGH, human growth hormone.
Materials and Methods

Peptide synthesis
Sequences of calcitonin, hGH, IFN-β, insulin, and Epo were obtained from the Swiss-Protein Database (http://www.expasy.org). Peptides spanning the entire sequences of salmon calcitonin (aa 83–114 of P01263), human Epo (aa 28–193 of P01588), hGH 1 isoform 1 (aa 27–217 of P01241), human insulin α (aa 90–110 of P01308), and human insulin β (aa 25–54 of P01308) were synthesized in house or purchased as crude material from Minotopes or Pepscan. Peptides synthesized in house were typically purified to >95% homogeneity by reverse-phase HPLC, and their identity was verified by mass spectrometry. Peptides for each of the test proteins were produced as 15mers overlapping by 10 aa.

Assays for peptide binding to purified HLA molecules
Quantitative assays to measure the binding of peptides to solubilized HLA molecules were performed by ELISPOT. In the case of the labeled standard peptide as previously described (25). We measured binding affinity of each peptide used in the study against 15 different MHC class II molecules including: DRB1*0101, DRB1*1501, DRB1*0301, DRB1*0401, DRB1*0404, DRB1*0405, DRB1*0701, DRB1*0802, DRB1*0901, DRB1*1101, DRB1*1302, DRB3*0101, DRB4*0101, DRB5*0101, DQB1*0301, and DQB1*0302. These class II molecules were chosen to be representative of most common HLA class II Ags worldwide. Specifically, the DRB1*0101, DRB1*1501, DRB1*0301, DRB1*0401, DRB1*1101, DRB1*0501, DRB1*0401, and DRB3*0101 allelic forms were chosen because each represents the most prevalent molecule of the DR1, DR2, DR3, DR4, DR11, DR51, DR53, and DR52a Ags, respectively. In the case of the DR7 Ag, only the DRB1*0701 allele was included in our panel because DRB1*0701 and DRB1*0702 vary in the C′ region which is outside the binding groove. In the case of the DR4 Ag, in addition to DRB1*0401, the DRB1*0405 allele was also studied because of its high prevalence in Asian populations. For DR8, the DRB1*0802 allele was chosen because it is the dominant allele in the majority population and DRB1*0802 and DRB1*0803 have nearly identical binding specificities. The DRB1*0901 allele was chosen as representative of the DR9 Ag as most of the allele variants are associated with silent mutations. The DRB1*0101 allele was typically chosen to compare the binding of the DR12 because DRB1*1201 and DRB1*1202 are evenly distributed. These alleles differ at position 67, which is not in the region of the binding groove and hence would not be predicted to strongly impact peptide binding. Finally, the DRB1*1302 allele was chosen to represent DR13 because this allele is slightly more prevalent than DRB1*1301. These HLA-DR types were chosen as representative of the most common HLA-DR phenotypes (11).

Generation of T cell lines
Human HTL cell lines were generated against wild-type Epo protein (R&D Systems) using primary in vitro immunization and PBMC from normal volunteers. As the source of PBMC, leukopheresis units from healthy donors were obtained through the General Clinical Research Center at The Scripps Clinic. HLADR-types were chosen as representative of the most common HLA-DR binding. In the case of the AT cells, HLA-DR molecules were based on the inhibition of binding to a radiolabeled standard peptide as previously described (25). We measured binding affinity of each peptide used in the study against 15 different MHC class II molecules including: DRB1*0101, DRB1*1501, DRB1*0301, DRB1*0401, DRB1*0404, DRB1*0405, DRB1*0701, DRB1*0802, DRB1*0901, DRB1*1101, DRB1*1302, DRB3*0101, DRB4*0101, DRB5*0101, DQB1*0301, and DQB1*0302. These class II molecules were chosen as representative of most common HLA class II Ags worldwide. Specifically, the DRB1*0101, DRB1*1501, DRB1*0301, DRB1*0401, DRB1*1101, DRB5*0101, DRB4*0101, and DRB3*0101 allelic forms were chosen because each represents the most prevalent molecule of the DR1, DR2, DR3, DR4, DR11, DR51, DR53, and DR52a Ags, respectively. In the case of the DR7 Ag, only the DRB1*0701 allele was included in our panel because DRB1*0701 and DRB1*0702 vary in the C′ region which is outside the binding groove. In the case of the DR4 Ag, in addition to DRB1*0401, the DRB1*0405 allele was also studied because of its high prevalence in Asian populations. For DR8, the DRB1*0802 allele was chosen because it is the dominant allele in the majority population and DRB1*0802 and DRB1*0803 have nearly identical binding specificities. The DRB1*0901 allele was chosen as representative of the DR9 Ag as most of the allele variants are associated with silent mutations. The DRB1*0101 allele was typically chosen to compare the binding of the DR12 because DRB1*1201 and DRB1*1202 are evenly distributed. These alleles differ at position 67, which is not in the region of the binding groove and hence would not be predicted to strongly impact peptide binding. Finally, the DRB1*1302 allele was chosen to represent DR13 because this allele is slightly more prevalent than DRB1*1301. These HLA-DR types were chosen as representative of the most common HLA-DR phenotypes (11).

Bioinformatic and structural modeling analysis
Structural modeling analysis on the Epo epitope analogues was performed to predict those likely to be least disruptive to the structural integrity of the Epo protein. The analysis was completed in three steps. First, we reviewed all available structure/function data to determine whether bioactivity of any of the Epo analogues detailed herein were reported in literature (30–33). Second, analyses of evolutionary conservation of epitope sequences were performed to determine residues that were polymorphic across species and, therefore, may be more amenable to substitution. The primary amino acid sequences for 24 mammalian Epo were aligned using BLASTP 2.2.3 (34). These included sequences representing human, macaque, cat, dog, cow, pig, sheep, rabbit, mouse, and rat proteins. Epo proteins were used as the query sequence. To determine structural conservation of residues, proteins that are structural neighbors to Epo were aligned using families of structurally similar proteins (35) and were analyzed similarly. These sequences included Epo (1IEE.A, 1CN4.C, 1BUY.A), ciliary neurotropic factor (1CN3.T), IL-6 (1ALU.A), stem cell factor (1EXZ.A), growth hormone (1HUW.A), G-CSF (1BGC.A), M-CSF (1ICF.A), leukemia inhibitory factor (1LDV.A), insulin (1EMP.A), and insulin-like growth factor (1IEVS.A), and GM-CSF (2GMF.A). Finally, molecular modeling was performed using published structures of the Epo molecule. The 1IEE.A structure was selected as the modeling template due to its high resolution and low R values, especially in the Epo protein domains that were selected for study. Both published and experimental variants were modeled using the Epo structure 1IEE.A as the template, Swiss-Model for modeling, and Deep View and WHATIF for subsequent analysis (36–39).

Expression and production of modified Epo proteins
DNA constructs encoding Epo variants carrying four different amino acid substitutions designated G2 (L102P, S146D), G3 (T107D, S146D), G4 (L102G, T107D, S146D), and G5 (L102S, T107D, S146D) were generated. Specific Epo variants were constructed by introducing specific substitutions in the wild-type Epo sequence using overlapping oligonucleotides encoding the analog mutations in a PCR assay. Blocks with 20-nt overlaps were generated for each analog and annealed together. These were then extended in a gene synthesis reaction (94°C for 30 s, 58°C for 30 s, and 72°C for 1 min for 5 cycles; 94°C for 30 s and 72°C for 1 min for 10 cycles) using the proofreading polymerase Pfu (Strategene). The extended blocks were amplified by PCR (94°C for 30 s, 58°C for 30 s, and 72°C for 1 min for 30 cycles) to synthesize full-length Epo analogues. Gel-purified DNA analog PCR products were cloned into pcDNA3 (Invitrogen Life Technologies) and confirmed by sequence analysis. The restriction sites HindIII and BamHI were engineered into the 5′ and 3′ ends, respectively, of each construct; the constructs were then subcloned into a mammalian expression vector. For expression of the analoged Epo sequences, HEK-293 cells were plated at a density of 2 × 10^6 cells per 100-mm polystyrene-coated cell culture dish in complete RPMI medium. The following day, cells were washed with RPMI without any antibiotics or serum and were transfected with 9 μg of plasmid DNA complexed to GenePorter liposome formulation (Gene Therapy Systems). Specifically, plasmid DNA...
and 45 μl of liposome were each diluted into 0.5 ml of medium and then combined. After allowing DNA to complex to the liposomes for 20 min at room temperature, the liposome DNA mixture was added directly to the washed cells. Control wells that did not contain any DNA were also included. After adding an additional 4 ml of complete medium, the cells were incubated at 37°C and 5% CO2 for 3 h. Following incubation, cells were washed once with complete RPMI medium and incubated for 72 h to allow for Epo expression. Cell supernatants were concentrated 6-fold by centrifugation at 2000 rpm using an Allegra 6KR centrifuge (Beckman Coulter) with an Amicon Centriprep (YM-10) ultracentrifugation device (Millipore).

### Quantitation of Epo variants

ELISA plates were coated overnight at 4°C with either anti-Epo mAB287 or mAB2871 (R&D Systems) at 10 μg/ml in bicarbonate buffer. Plates were subsequently washed and then blocked (10% FBS in PBS) for 1 h. Cell expression supernatants were added at varying dilutions to the ELISA plate and incubated for 2 h at room temperature. Standard curves were generated using purified recombinant Epo (R&D Systems). Following sample incubation, plates were washed and rabbit anti-Epo Ab (R&D Systems) was applied at 1/1000 for 1 h at room temperature. Finally, anti-rabbit HRP-conjugated Ab (Clontech Laboratories) at the dilution of 1/5000 was applied for 1 h. The plates were then washed and substrate solution was added for 1 h. The absorbance at 450 nm was measured and used to calculate the concentration of the sample.

### Table I. Binding analysis of overlapping peptides from Epo, calcitonin, human growth hormone, IFN-β, and insulina

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Binding (nM)</th>
<th>a</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epo</td>
<td>495</td>
<td>500,000</td>
<td>100</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>450</td>
<td>50,000</td>
<td>100</td>
</tr>
<tr>
<td>Human growth hormone</td>
<td>400</td>
<td>50,000</td>
<td>100</td>
</tr>
<tr>
<td>IFN-β</td>
<td>350</td>
<td>50,000</td>
<td>100</td>
</tr>
<tr>
<td>Insulin</td>
<td>300</td>
<td>50,000</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>a</th>
<th>Indicates IC50 &gt; 50,000 nM.</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>The first amino acid position of the overlapping synthetic peptide. Number of HLA molecules bound at 1,000 nM.</td>
</tr>
</tbody>
</table>

---

*The first amino acid position of the overlapping synthetic peptide. Number of HLA molecules bound at 1,000 nM.*
added and incubated for 1 h. Plates were developed using HRP substrate (Kirkgegaard & Perry Laboratories). Color was measured at OD450 using a Labsystems Multiscan RC plate reader.

Bioactivity of modified Epo proteins

The bioactivity of modified Epo proteins was measured in a cell proliferation assay using the erythroleukemic cell line, designated TF-1, which is dependent on GM-CSF, IL-3, and Epo for growth (40, 41). The TF-1 cells were cultured in 10 ng/ml recombinant human GM-CSF. On the day before performing the assay, the cells were washed and resuspended in RPMI 1640 medium containing no growth factors. A total of 3 × 10^4 of these cells were plated in 96-well flat-bottom plates and incubated with various dilutions of wild-type Epo protein or concentrated supernatants from cells transfected with analog proteins. The cells were incubated at 37°C for 96 h and pulsed with 2 radioactivity. To confirm the specificity of the bioassay, the Epo-specific response was blocked by preincubation of the supernatants or wild-type Epo with a polyclonal anti-Epo Ab.

GM-CSF induced proliferation was not blocked using the same anti-Epo Abs.

For testing immunogenicity of proteins, DC were pulsed with culture supernatants from DNA constructs transfected with either wild-type Epo or modified Epo proteins G3 and G4. Following two in vitro restimulations with peptide or protein-pulsed PBMC, cultures were tested for peptide-specific responses using the IFN-γ-based ELISPOT assay. Five different donors, whose CD4^+ T cells had responded to Epo, were tested. The data are expressed as the total number of positive cultures (using the positive ELISPOT criteria described above) per number of cultures tested, to represent frequency of responses, and as number of net SFC per 5 × 10^5 effector cells to represent response magnitude.

Results

HLA class II binding capacity of candidate proteins

Overlapping 15-aa synthetic peptides spanning the entire sequence of calcitonin, human Epo, hGH, human insulin, and human IFN-β were tested for binding to a panel of 15 HLA-DR molecules (Table I). We found that most of the peptides bound one or more HLA-DR molecules; only 12% of the peptides did not bind any of the HLA-DR molecules tested (Table I). These data suggest that generation of proteins completely devoid of immunogenic potential maybe an unrealistic goal. Therefore, we reasoned that a more practical alternative for reducing immunogenicity was to identify degenerate immunodominant epitopes and to modify them accordingly.

To illustrate the relation between immunodominance and degenerate binding, 29 known HLA-DR epitopes were synthesized and tested for binding to 15 different HLA class II molecules. The majority of the reported restriction events, 32 of 48 (67%), were associated with degenerate binding, which was defined as the capacity to bind 40% (6 of 15) of the HLA-DR molecules tested (data not shown).

The degenerate binding regions from the therapeutic proteins insulin, IFN-β, hGH, and Epo are shown in Table II. Although insulin and calcitonin did not contain any degenerate binding peptides, several highly degenerate regions were identified within Epo, hGH, and IFN-β. Four main degenerate binding regions were identified for hGH between residues 8 and 22, 71 and 106, 134 and 159, and 245 and 270. For Epo, 101–115 and Epo (136–150) in the form of synthetic peptides or Epo epitope analog combinations C2 (L102P and S146D), C3 (T107D and S146D), C4 (L102G, T107D, and S146D), and C5 (L102S, T107D, and S146D). CD4^+ T cells were expanded in vitro after two restimulations and then tested for IFN-γ production by the ELISPOT assay. For testing immunogenicity of proteins, DC were pulsed with culture supernatants from DNA constructs transfected with either wild-type Epo or modified Epo proteins G3 and G4. Following two in vitro restimulations with peptide or protein-pulsed PBMC, cultures were tested for peptide-specific responses using the IFN-γ-based ELISPOT assay. Five different donors, whose CD4^+ T cells had responded to Epo, were tested. The data are expressed as the total number of positive cultures (using the positive ELISPOT criteria described above) per number of cultures tested, to represent frequency of responses, and as number of net SFC per 5 × 10^5 effector cells to represent response magnitude.
To precisely identify the epitopes recognized, HTL lines from five donors were tested. For each individual we calculated the magnitude of response against each peptide using our panel of 36 overlapping Epo peptides. In Fig. 1a, we show the cumulative SFCs from all five donors against each peptide, shown in the line, compared with the number of HLA-DR molecules bound for each peptide, shown in the bars. Using these data, we mapped two highly immunogenic regions to residues 91–120, containing three overlapping peptides (91–105, 96–110, and 101–115), and residues 126–155, containing two overlapping peptides (131–145 and 136–150). In two donors, responses were obtained against Epo peptides 51, 68, 86, and 106. However, the magnitude of these responses was significantly lower than those obtained to regions 91–120 and 126–155.

In Fig. 1b, we assessed the frequency of donors responding to each peptide. The line depicts the percentage of donors responding to each peptide compared with the number of HLA-DR molecules bound for each peptide, shown in the bars. The highest frequency of response (80–100%) was detected against peptides corresponding to Epo position numbers 91, 101, and 136, while fewer donors responded against several other peptides. Thus, the most degenerate Epo peptides in regions 91–120 and 126–155 were the most frequently recognized peptides recognized by different donors, also known as immunodominant epitopes.

**Epitope analogues with reduced HLA degeneracy and antigenicity**

We selected the most immunogenic and HLA-DR degenerate Epo peptides, designated as Epo 101 and Epo 136, for further analysis. A total of 100 different analogues of the two wild-type peptides Epo 101 and 136 were tested for binding to the 15 HLA-DR molecules. Twelve analogues of the Epo 101 epitope and nine analogues of the Epo 136 were identified with a 10-fold reduction in binding affinity for at least five HLA-DR molecules (Table IV). Analogues with the most significant reduction in binding capacities were those with double substitutions. For example, the binding affinity of the GGRSLTDLRLGAQ double analogue was reduced by 10-fold or greater for 13 of 15 and 14 of 15 HLA-DR, respectively (Table IV, Epo 101) (bold

### Table III. Immunogenicity in vitro of wild-type Epo protein with PBMC from normal donors

<table>
<thead>
<tr>
<th>Donor</th>
<th>Class II MHC Type</th>
<th>Erythropoietin Specific Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DRB1*(1501, 0701), DRB4* (0101), DRB5* (0101)</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>DRB1*(0101, 0405), DRB4* (0101)</td>
<td>Y</td>
</tr>
<tr>
<td>3</td>
<td>DRB1*(1301, 0801), DRB3* (0101)</td>
<td>Y</td>
</tr>
<tr>
<td>4</td>
<td>DRB1* (0301, 1101), DRB3* (0101)</td>
<td>Y</td>
</tr>
<tr>
<td>5</td>
<td>DRB1* (0901, 1406), DRB3* (0101), DRB4* (0101)</td>
<td>Y</td>
</tr>
<tr>
<td>6</td>
<td>DRB1* (0301, 0701), DRB3* (0101), DRB4* (0101)</td>
<td>Y</td>
</tr>
<tr>
<td>7</td>
<td>DRB1* (0101)</td>
<td>N</td>
</tr>
</tbody>
</table>

* Y indicates that a positive cell line was obtained and N indicates that a positive cell line was not obtained.

* A 10-fold reduction in binding affinity for at least five HLA-DR molecules was detected.

47, and 155 and 169. In the case of IFN-β, five degenerate binding regions were identified between residues 6 and 20, 16 and 30, 56 and 80, 80 and 111 and 140, and 144 and 166.

Four main degenerate binding regions were identified in Epo and are located between residues 46 and 65, 66 and 80, 91 and 120, and 126 and 155. Each degenerate region contained either one or more overlapping peptides. For each region, the most degenerate peptides were as follows: peptide 51–65 binding 8 of 15 molecules, peptide 66–80 binding 7 of 15 molecules, peptide 101–116 binding 11 of 15 molecules, and peptide 136–150 binding 11 of 15 molecules. In conclusion, degenerate regions were identified for three of five proteins analyzed.

**Immunogenicity of wild-type Epo protein and antigenicity of Epo peptides in vitro**

We selected Epo as a model protein and examined its in vitro immunogenicity using human PBMC obtained from normal donors. For these in vitro studies, PBMC were obtained from seven normal donors with a diverse set of MHC haplotypes. Epo-specific HTL responses were induced in five of seven (71%) of the donors tested (Table III). These data clearly demonstrate the immunogenicity of Epo across various HLA-DR types.

---

![Correlation between antigenicity and HLA binding degeneracy of overlapping Epo peptides: Magnitude of Response](image-url)

**FIGURE 1.** Correlation between antigenicity and HLA binding degeneracy of overlapping Epo peptides. Purified CD4+ T cells from normal donors were used to derive HTL lines from each donor using ELISPOT assays. Overlapping peptides spanning the entire Epo sequence were assayed for antigenicity against HTL lines from each donor using ELISPOT assays. The first position of each peptide is shown on the x-axis. The number of HLA molecules bound at ≤1000 nM by each peptide is indicated on the secondary y-axis as a line graph. a. The cumulative SFC responses measured against each individual peptide are shown on the primary y-axis and are depicted as bar graphs. b. The percentages of responding donors against each individual peptide are shown on the primary y-axis as bar graphs.
letters represent amino acid changes from the original Epo sequences). The binding affinity of the corresponding single substitution analogues at position 2 was reduced for 7 of 15 (S analog) or 6 of 15 (G analog) HLA-DR molecules. A significant reduction in the number of HLA-DR bound at the 1000-nM level was also observed for the GSRSL.TDLRLAGQ and GGRSL.TDLRLAGQ analogues, for which binding was reduced to 3 of 15 or 2 of 15 HLA-DR molecules, respectively. The corresponding single position 2 substituted analogue bound to 8 of 15 molecules (Table IV, Epo 101). Similar results were obtained with the double analog

### Table IV. Epo analogs with reduced binding capacity

<table>
<thead>
<tr>
<th>Epo 101</th>
<th>Binding capacity (IC50 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLRSL.TDLRLAGQ</td>
<td>8.5</td>
</tr>
<tr>
<td>GERSLTTLRRLAGQ</td>
<td>45</td>
</tr>
<tr>
<td>GRSRL.TDLRLAGQ</td>
<td>31</td>
</tr>
<tr>
<td>GGRSL.TDLRLAGQ</td>
<td>12</td>
</tr>
<tr>
<td>GRSRL.TDLRLAGQ</td>
<td>213</td>
</tr>
<tr>
<td>GRSRL.TDLRLAGQ</td>
<td>79</td>
</tr>
<tr>
<td>GLRSL.TDLRLAGQ</td>
<td>90</td>
</tr>
<tr>
<td>GLRSL.TDLRLAGQ</td>
<td>69</td>
</tr>
<tr>
<td>GLRSL.TDLRLAGQ</td>
<td>15</td>
</tr>
<tr>
<td>GURSBTTLRRLAGQ</td>
<td>44</td>
</tr>
</tbody>
</table>

### Table V. Antigenicity of Epo analogs with reduced binding capacity

<table>
<thead>
<tr>
<th>Epo 136</th>
<th>Binding capacity (IC50 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTFRKDFRVYSLNR</td>
<td>12</td>
</tr>
<tr>
<td>DTFRKDFRVYSLNR</td>
<td>148</td>
</tr>
<tr>
<td>DTFRKDFRVYSLNR</td>
<td>98</td>
</tr>
<tr>
<td>DTFRKDFRVYSLNR</td>
<td>54</td>
</tr>
<tr>
<td>DTFRKDFRVYSLNR</td>
<td>196</td>
</tr>
<tr>
<td>DTFRKDFRVYSLNR</td>
<td>11</td>
</tr>
<tr>
<td>DTFRKDFRVYSLNR</td>
<td>76</td>
</tr>
<tr>
<td>DTFRKDFRVYSLNR</td>
<td>140</td>
</tr>
<tr>
<td>DTFRKDFRVYSLNR</td>
<td>144</td>
</tr>
<tr>
<td>DTFRKDFRVYSLNR</td>
<td>11107</td>
</tr>
</tbody>
</table>

a Wild-type peptides are underlined.

b No binding observed.

c The number of MHC molecules to which binding capacity was reduced by 10-fold or more.

d Frequency of MHC molecules to which peptide binds at IC50 ≤ 1000 nM.

- Table IV. Epo analogs with reduced binding capacity

- Table V. Antigenicity of Epo analogs with reduced binding capacity

---

a Wild-type peptides are underlined.

b The predicted impact of various substitutions on the Epo protein bioactivity was determined as described in detail in Materials and Methods.

c Antigenicity as measured by the IFN-γ ELISPOT assays using T cell lines generated with wild-type Epo. Responses were scored on the basis of the percent residual antigenicity as compared to wild-type Epo peptides. The four classes used were: + + + (70–90%), + + (40–70%), + (10–40%), − (≤ 10%) of the wild-type peptide response.

d Frequency of disruption indicates the frequency of donors in which residual antigenicity was 40% or less.
DTFRKD FRVYD NFLR of the Epo 136 epitope (Table IV, Epo 136) where the binding capacity was reduced by 10-fold or greater in 12 of 15 molecules. These results compare favorably with those from corresponding single substitution analogues where the binding capacity was reduced in 8 of 15 (position 6 substituted) or 5 of 15 (position 11 substituted) molecules. In fact, the double analog bound to only 3 of 15 MHC molecules, compared with the corresponding single analogues, which bound 9 of 15 or 7 of 15 molecules when substituted at position 6 and position 11, respectively.

The antigenicity of these epitope analogues was assessed using HTL lines specific for the whole Epo protein, derived from four different donors (Table V). Single amino acid substitutions associated with the greatest reductions in binding affinity were recognized least by HTL lines. For example, responses to peptides GLRSLTLRALGAQ, GPRSLLTTLRALGAQ, DTFRKLFRVYDNFLR, and DTFRKD FRVYSNFLR were disrupted by >60% in >75% of the donors. As predicted, the double analogues GSRSLTLTLRALGAQ, GGRSLTLTLRALGAQ, and DTFRKD FRVYDNFLR were recognized to an even lesser degree, with decreases in magnitude ≥90% in ≥75% of donors tested.

**Molecular modeling of Epo analogues**

Next, we analyzed which domains and residues may be important for Epo bioactivity. Our analysis indicated that 70% of substitutions for amino acids R103, S104, R143, and N147 located within the Epo receptor contact sites, result in decreased bioactivity compared with just 6% for substitutions of amino acids outside these contact sites. The amino acid substitutions contained within the Epo epitopes Epo 101–115 (GLRSLTTLLRALGAQ) and Epo 136–150 (DTFRKLFRVYSNFLR) at positions L102, R103, S104, L105, T107, L141, R143, V144, S146, and N147 were analyzed further, as these residues were in close proximity to the described receptor contact sites and could have the most structural impact. In the primary mammalian sequences, little conservation was seen for S104. Residues L105, T107, V144, and S146 were variable although substitutions at these positions were generally quite conservative. Moderate conservation was seen for R103 and T107. Comparison of structural neighbors for the five residues in peptide Epo 101–115 showed that L102 was very well conserved, with most residues in the structural neighbors preserving the aliphatic nonpolar nature of this position. For experimental variants within the peptide Epo 136–150 (DTFRKLFRVYSNFLR), all residues except V144 were well conserved in the structural neighbors.

Experimental Epo variants were modeled using the Epo structure 1EER:A as the template structure for model building. With the exception of the substitution of P at L102 (root mean square deviation of 0.55 Å) the proposed experimental substitutions had little effect on the gross predicted structures of the Epo variant proteins, with root mean square deviations for the main backbone of 0.14 Å or less.

Based on this analysis we predicted that with the possible exceptions of L→P102 and N→D147 which are located within the receptor contact site, all other epitope analogues analyzed in the previous section should result in Epo variants that retained wild-type bioactivity in vivo (Table V).

**Immunogenicity of Epo 101 and Epo 136 epitope analogues with reduced binding capacities**

To confirm that the Epo epitope analogues with disrupted HLA-DR binding affinity were less immunogenic, we tested peptide combinations in primary in vitro immunogenicity assays using PBMC from five donors that had responded previously to whole wild-type Epo and to wild-type Epo epitopes Epo 101 and Epo 136. Cells were expanded in vitro after two restimulations. In each case, one Epo 101 analog and one Epo 136 analog were tested.
after pooling equimolar concentrations. Specifically the Epo analog combinations tested were C2 (L102P and S146D), C3 (T107D and S146D), C4 (L102G/T107D and S146D), and C5 (L102S/T107D and S146D) was measured in a cell proliferation-based assay using the TF-1 cell line. Wild-type Epo and supernatants from untransfected cells were tested as positive and negative controls, respectively. The data are represented as stimulation index.

The mean response against the wild-type combination was 319 SFC/5 × 10⁴ effector cells. Significant responses were noted for most donors, with donor no. 6 being the exception and not responding in this particular experiment to either the wild-type or the analog peptides. The analog epitope combinations were significantly less immunogenic with mean values for the peptide combinations C2, C3, C4, and C5 of 20, 86, 45, and 29 SFC/5 × 10⁴ effector cells, respectively (p < 0.005). With respect to frequency of responses, the wild-type peptide combination yielded 36 of 50 positive cultures whereas the analog C2, C3, C4, and C5 yielded 9 of 50, 12 of 50, 6 of 50, and 14 of 50 positive cultures, respectively (p < 0.005).

**Expression, production, and bioactivity of modified Epo protein**

Modified Epo proteins containing substitutions corresponding to epitope analog combinations tested above, named G2, G3, G4, and G5 were produced next. All these modified proteins were detected by western blot using polyclonal Abs. However, the protein G2 was not detected by ELISA. Two different Epo Abs were used in this ELISA to ensure that the lack of detection of G2 was not due to the lack of expression but due to loss of reactivity with a particular Ab. Therefore, we believe that our inability to detect the G2 form of the protein using mAbs reflects a gross structural alteration associated with the L→P substitution at position 102. The amount of modified Epo protein contained in each culture supernatant was quantitated using recombinant wild-type Epo protein. The levels varied from 165 U/ml for G3, 211 U/ml for G4, and 152 U/ml for G5 (data not shown); these differences were not considered to be significant.

Bioactivity of the modified Epo constructs was analyzed next (Fig. 3). Supernatants from G3 and G4 modified proteins were bioactive in the same range as that of the commercially available wild-type Epo protein. However, we could not detect any bioactivity for the G5 protein supernatant. Thus the L→S change at position 102 appears to significantly alter functional activity.
Immunogenicity of bioactive modified Epo proteins

The immunogenicity of wild-type Epo and the modified Epo proteins G3 and G4, was tested using PBMC from the same five donors previously used (Fig. 4). The data were evaluated in terms of both magnitude and frequency of response. The immunogenicity of proteins G3 and G4 was lower than that of wild-type Epo protein, with a mean response of 6 and 9 SFC/5 × 10^4 effector cells for modified proteins G3 and G4 as compared with a mean response of 44 SFC/5 × 10^4 effector cells for the wild-type protein (p < 0.001). The frequency of cultures generated using the modified Epo was also lower, with 9 of 40 and 8 of 40 positive cultures for G3 and G4 proteins, respectively, vs 27 of 40 positive cultures for wild-type protein. As expected, supernatants from untransfected cells were not immunogenic. In conclusion, our data demonstrate that the intrinsic immunogenicity of Epo can be reduced by modification of immunodominant HTL epitopes while retaining bioactivity.

Discussion

We developed an integrated approach to reduce the intrinsic immunogenicity of therapeutic protein drugs. The approach is based on 1) the identification of immunodominant epitopes and 2) reducing their binding affinity to HLA-DR molecules to levels below those associated with naturally occurring HTL epitopes. Our approach is based on quantitative determination of HLA-DR binding affinity coupled with confirmation of these epitopes by in vitro immunogenicity testing. These quantitative measurements help in determining the overall intrinsic immunogenic potential of a protein. We are aware that immunogenicity of a protein is dependent not only on intrinsic immunogenic potential encoded in HLA binding characteristics but also on several extrinsic factors, such as formulation, dose, and/or route of administration. Our approach does not address these issues, but rather it does provide a means to objectively assess the overall intrinsic immunogenicity potential of a protein and ways to reduce this intrinsic immunogenicity. As such, our approach is a broadly applicable tool as a first fundamental step in determining the immunogenic potential of therapeutic proteins and judging whether modifications are required.

In initial experiments the binding capacity of 105 overlapping synthetic peptides spanning the full-length sequences of Epo, hGH, insulin, calcitonin, and IFN-β were evaluated to determine the predicted immunogenic potential of these drugs in a population of diverse ethnic backgrounds. These 105 peptides were tested for binding to a panel of 15 HLA molecules representative of the main HLA-DR Ags worldwide (11). To the best of our knowledge, this is the most comprehensive analysis of this type completed when HLA polymorphism is considered.

The results of the binding analysis showed that 88% of the peptides bound to one or more of the HLA molecules tested, suggesting that complete elimination of HLA-DR binding capacity is unrealistic and that generation of proteins completely devoid of any immunogenic potential not be a practical goal. The high number of substitutions required would necessitate a laborious modification process and would essentially result in the generation of a new protein. In addition, the modified new protein potentially would have high nonself content and, thus, increased intrinsic immunogenic potential and poorly predictable biological activity. However, binding analysis does provide a means to identify immunodominant epitopes with degenerate binding patterns. Modification of these epitopes to reduce immunogenicity, might be a realistic and practical approach.

To identify immunodominant epitopes, we analyzed five different therapeutic proteins for which evidence of immunogenicity exists in humans, namely, hGH, calcitonin, insulin, IFN-β, and Epo (5, 6, 24, 42, 43). Although insulin and calcitonin did not contain any clear degenerate binding regions, four, six, and four degenerate binding regions were identified within the hGH, IFN-β, and Epo proteins, respectively. This result suggested that the intrinsic binding capacity of these proteins might be reduced with a relatively small number of substitutions. We selected Epo as a model Ag for detailed studies because functional and biological assays to test bioactivity of this molecule are available and based on the existence of a large structural database for Epo.

The immunogenicity of the intact Epo protein was evaluated using primary in vitro immunogenicity assays using PBMC from normal healthy blood donors. We used in vitro human primary assays rather than in vivo animal models, because most candidate therapeutic proteins are human proteins and would be expected to be recognized as nonself in most laboratory animals. Moreover, the types of MHC molecules expressed in animal models are associated with a peptide binding specificity different from human MHC, resulting in a different set of epitopes being presented. Our in vitro immunogenicity experiments used PBMC from a limited number of donors. However, we emphasize that because actual binding determinations against HLA-DR molecules representative of the worldwide population were performed, any bias resulting from sampling populations with a narrow ethnic spread was avoided.

Our results demonstrate that, at least in the case of Epo, immunodominant epitopes correspond to the most degenerate HLA-DR binding regions. These findings support earlier reports that describe a relationship between immunodominance and degenerate binding (18–21). Thus a few immunodominant epitopes with degenerate binding could be targeted for the purpose of reducing the immunogenicity of protein drugs. Introducing as few substitutions as possible is highly desirable because each substitution could impact bioactivity or create a neoepitope, by introducing a nonself residue in the sequence of the self-protein.

Several analogues that are associated with significantly reduced binding capacity, antigenicity, and immunogenicity were identified. To accomplish this goal, an analoging strategy was developed using D, R, and G substitutions at nonanchor positions in addition to avoiding aromatic or hydrophobic residues at the P1 anchor position. This strategy greatly reduced the number of analogues to be generated for each epitope. Because of its general applicability, we anticipate that this strategy might be of use for the generation of other protein Ags with reduced immunogenicity.

Structure/function data, sequence conservation and structural modeling analysis was also used to predict the impact of the substitutions on the biological activity of the Epo protein. These studies illustrated how bioinformatic structural analysis can assist in the design of proteins with reduced immunogenicity and unaffected biological and pharmacological functions.

When the immunogenicity of the two modified Epo proteins was compared with that of wild-type protein, reduced HLA-DR binding affinity values were associated with reduced immunogenicity. Although it is still possible that such modified protein drugs might be immunogenic and that immune responses against subdominant epitopes might develop, we predict that such responses will be of lower frequency and/or magnitude because the intrinsic immunogenic potential of the protein has been reduced. In particular, our analysis did not address the potential immunogenicity associated with DP and DQ molecules. Studies are currently ongoing in our laboratory to expand our analysis to include common allelic variants of these isotypes.
In summary, this is the first demonstration of an integrated, systematic approach for reducing immunogenicity of therapeutic proteins. Immunodominant epitopes were identified by high throughput in vitro HLA-DR binding assays for a panel of HLA allele variants, representative of the worldwide polymorphisms in conjunction with in vitro cellular immunogenicity assays with human PBMC. Epitope identification was followed by rational modification of these epitopes to reduce their HLA-DR binding capacity, verification of bioactivity, and verification of reduced immunogenicity of analog proteins. Our findings validate this strategy for engineering safer protein drugs and provide a rationale to evaluate in the clinical setting the proteins defined according to this procedure.

Acknowledgments
The expert secretarial help of Kelly Riddle-Hilde is gratefully acknowledged.

Disclosures
The authors have no financial conflict of interest.

References