Peptide Immunogen Mimicry of a Protein-Specific Structural Epitope on Human Choriogonadotropin

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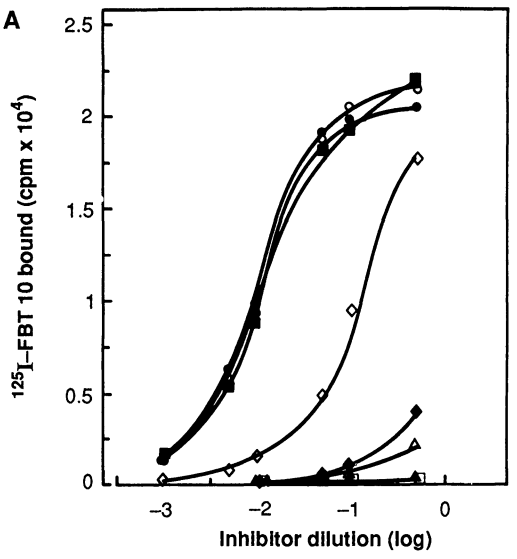
It is a challenge to construct synthetic immunogens that elicit antibodies (Abs) both directed to conformational epitopes and specific for a complex protein like human choriogonadotropin (hCG). A monoclonal antibody specific for hCG bound to regions around Lys[45] of the α subunit (hCGα) and Asp[112] of the β subunit (hCGβ). A peptide comprising residues 46 to 55 of hCGα and residues 106 to 116 of hCGβ elicited Abs in rabbits that were directed to a discontinuous epitope and were specific for hCG. These Abs inhibited the binding of hCG to its receptor. Thus, a synthetic immunogen can mimic a conformational-specific epitope and can be useful for vaccine development.

THE USE OF SYNTHETIC PEPTIDES AS vaccines (*1*) has been hampered because most short peptides mimic continuous epitopes composed of a linear stretch of residues in the antigen, whereas most epitopes that can elicit Abs are probably in antigens with intact tertiary or quaternary structures (*2*). These discontinuous (or conformational) epitopes comprise amino acids that are not consecutive in sequence but are juxtaposed by peptide folding or by quaternary structure. Attempts have been made to develop antifertility synthetic vaccines by the use of peptides analogous to different portions of hCG (*3, 4*). This placental hormone is structurally related to the pituitary hormones lutropin (hLH), follitropin, and thyrotropin, which consist of two noncovalently associated subunits, α and β, where the α subunits are identical and the β subunits are quite similar. hCGβ and hLHβ are 82% identical, although hCGβ has a unique sequence of 24 amino acids at the COOH-terminus (*5*). A synthetic peptide of the COOH-terminal 37 amino acids of hCGβ coupled to diphtheria toxoid has been tested for vaccination, but is of poor antigenicity and immunogenicity (*4, 6*). Thus, other hCG-specific epitopes are needed to construct new synthetic peptide vaccines (*7*). Because of both the biochemical structure of hCG and the similarity of hCG and hLH, such hCG-specific immunogenic sites are probably discontinuous epitopes, so the peptides would have to mimic a tertiary or quaternary protein structure.

To localize an hCG-specific epitope, we had previously studied the discontinuous antigenic regions recognized by a monoclonal antibody (MAb) to hCG, C8, which binds only to hCG and does not cross-react with either free hCGα and hCGβ subunits or other glycoprotein hormones (*8*). C8 binds to a region of hCGβ including, or near to, Asp[112] (*9*). In a "two-site" monoclonal immunoradiometric assay (M-IRMA) (*10*), MAb C8 bound both to the hCGβ subunit and the α subunit of human, equine, porcine (*9*), ovine, turtle, and carp (Fig. 1A), indicating that C8 recognized a region of the α subunit conserved throughout evolution, most likely located near Lys[45] (*11*). Thus, the hCG-specific antigenic site recognized by C8 was probably a discontinuous epitope comprising amino acid residues located within the hCGα(41–60) (residues 41 to 60) and hCGβ(101–121) regions (Fig. 1B).

Various synthetic peptides (*12*), whose sequences included part or the totality of the antigenic site of C8, were then constructed to find peptide-carrier conjugates (*13*) that could elicit Abs specific for hCG. A first peptide (batch 1) spanning the hCGα(43–49)–hCGβ(110–116) regions was coupled to tetanus toxoid (TT) and injected into rabbits (Table 1A) (*14*). After four booster injections, Abs to peptide were detected with an enzyme-linked immunosorbent assay (ELISA) (*15*), but these Abs did not bind to either hCG or its subunits by radioimmunoassay (RIA) (*16*). Another peptide (batch 2), spanning longer peptide sequences and including the hCGα(43–55) and the hCGβ(106–116) regions, was cou-



B

		45	51
hCGα	(41-60)	: L R S K K T M L V Q K N V T S E S T C C	
oLHα	(45-64)	: A - - - - - - - - - P - - I - - - A - - -	
cGTHα	(45-64)	: A - - - - - - - - - P Q D I - - - A - - -	
			112
hCGβ	(101-121)	: G G P K D H P L T C D D P R F Q D S S S S	

Fig. 1. (**A**) Delineation of antigenic regions on the α subunit recognized by MAb C8 to hCG by recombination experiments. Briefly, α subunits from different species (4 μg) were recombined with hCGβ (5 μg) by incubation for 20 hours at 37°C (*9*). The final products were then diluted 1:2 to 1:1000 in normal human serum and tested in a two-step immunoradiometric assay based on C8 as "capture" antibody on a solid-phase support and ^{125}I-labeled monoclonal anti-hCGβ antibody FBT 10 as radiolabeled indicator (*10*). Results are expressed as ^{125}I-FBT 10 bound in counts per minute. The indicated values represent the arithmetic mean of duplicate samples taken from one experiment. The standard deviation (SD) were all less than 10% of the sample mean; and each experiment was reproduced at least twice with similar results. hCGα (♦), hCGβ (△), ovine LHα (▲), turtle LHα (□), carp GTHα–hCGβ (■), hCGα-hCGβ (○), ovine LHα–hCGβ (●), and turtle LHα–hCGβ (◇). (**B**) hCGα and hCGβ regions, including some amino acid residues contributing to the antigenic determinant of MAb C8, as suggested by both previous (*9*) and present experiments (Fig. 1A). The hCGα portion corresponds to a highly conserved region on α subunits of different species. Under the hCGα sequence are shown those residues of ovine LHα (oLHα) and carp GTHα (cGTHα) that differ from hCGα. The amino acid sequences are shown by means of the single-letter code (*24*).

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pled to TT and injected into rabbits under the same experimental conditions. Such a peptide-carrier conjugate elicited Abs that bound to both hCG and hCGβ as demonstrated by RIA. However, Abs to the α subunit and hLH were also present in one animal. In an attempt to avoid such cross-reactivities, we assembled the hCGβ(106–116) portion with either the short length hCGα(43–49) or the hCGα(46–55) sequence (batches 3 and 4, respectively). Abs were elicited by the hCGα(43–49)–hCGβ(106–116) peptide that cross-reacted with hLH in one antiserum, whereas Abs to the hCGα(46–55)–hCGβ(106–116) peptide were consistently specific for hCG, hCGβ, or both (Table 1A). The latter peptide-TT conjugate results were confirmed with other peptide-carrier conjugates (batches 5 to 7) based on the same peptide sequence, but with different coupling agents, carriers, or immunization doses (Table 1B). In 19 of 20 rabbits injected with 200 μg of peptide (batches 5 and 6) and 2 of 10 rabbits injected with a lower dose (15 μg) of peptide (batch 7), Abs to hCG were present; these antisera were consistently specific for hCG, hCGβ, or both. The ability of a peptide comprising an hCGα portion and the hCGβ(106–116) region to induce an hCG-specific Ab response depended on the hCGα sequence, because peptides that included Ser^{43}-Lys^{44}-Lys^{45} of hCGα (batches 2 and 3) elicited Abs that cross-reacted with hLH, whereas a peptide without these residues elicited Abs specific for hCG, hCGβ, or both.

Four (representative) antisera from rabbits injected with hCGα(46–55)–hCGβ(106–116) peptide-conjugates were titrated on hCG or the individual subunits (Fig. 2). One antiserum (R40-7) bound to hCG, but not to hCGβ or to the peptide. Lack of binding to the peptide in an ELISA might be due to modifications of the peptide structure in the immunizing preparation or in the solid-phase support (*17, 18*). The unique specificity of antiserum R40-7 for hCG with no binding to hCGβ, and the preferential binding of antiserum R9-4 to hCG in comparison to hCGβ, suggested that a population of Abs with a unique specificity for hCG might predominate in some antisera that are elicited by the hCGα(46–55)–hCGβ(106–116) peptide.

To characterize the antigenic sites recognized by Abs in the same four antisera, we tested free subunits or hybrids formed by recombination of an α subunit with a β subunit, for inhibition of ^{125}I-labeled hCG (^{125}I-hCG) binding to the antisera (*19*). Free hCGβ was as potent as the recombinant hCGα-hCGβ product in inhibiting binding to ^{125}I-hCG to both antisera R10-4 and R37-6, suggesting that most Abs in these antisera were to the hCGβ(106–116) portion (Fig. 3). In effect, inhibition profiles of such antisera were similar to those ob-

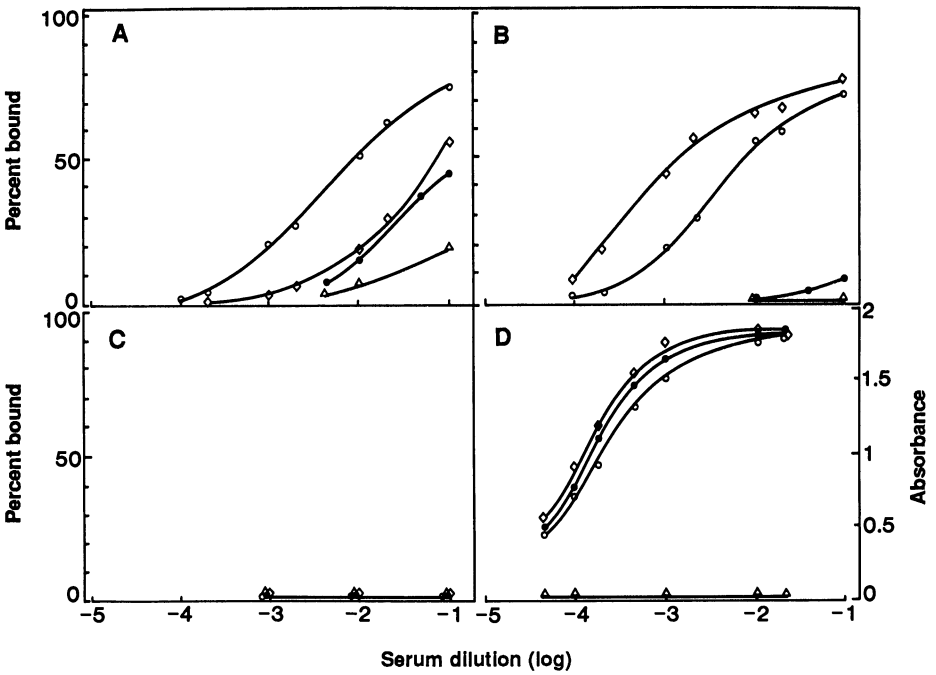

Fig. 2. Titration of antiserum R9-4 (●), R10-4 (◇), R37-6 (○), or R40-7 (△) collected from four rabbits injected with different batches of hCGα(46–55)–hCGβ(106–116) peptide-carrier conjugate. Binding of (**A**) ^{125}I-hCG, (**B**) ^{125}I-hCGβ, or (**C**) ^{125}I-hCGα to different antisera was tested by RIA (*16*) and expressed in percent of radiolabeled hormone or subunit bound. (**D**) Binding of the hCGα(46–55)–hCGβ(106–116) peptide to each respective antiserum was tested by ELISA (*14*). The indicated values represent the arithmetic mean of duplicate samples (as explained in Fig. 1). Standard deviation for each data point was <5%; and each experiment was reproduced at least twice with similar results. The first number identifies the rabbit eliciting the antiserum and the second indicates the batch of immunogen used to inject the rabbit, as indicated in Table 1.

Table 1. Antibody response of rabbits injected with different peptide-carrier conjugates varying in (A) peptide sequence and (B) carrier or coupling agent. Peptides depicted were synthesized according to the solid-phase method of R. B. Merrifield (*12*). The peptides were coupled to tetanus toxoid (TT) or keyhole limpet hemocyanin (KLH) with *N*-cyclohexyl-*N'*-morpholinoethyl carbodiimide (CMECDI), glutaraldehyde, benzidine, or tetramethylbenzidine as coupling agent (*13*). After immunizations (*14*), antisera were screened for specific reactivity by either ELISA with the respective noncoupled immunizing peptide (*15*) or RIA with ^{125}I-labeled hormones or subunits (*16*).

Batch	Peptide sequence	Coupling agent	Carrier	Number of rabbits	Number of rabbits with antibodies to				
					Peptide*	hCG†	hCGβ†	hCGα†	hLH†
A									
1	hCGα(43–49)–hCGβ(110–116)	CMECDI	TT	4	4	0	0	0	0
2	hCGα(43–55)–hCGβ(106–116)	CMECDI	TT	2	2	2	2	1	1
3	hCGα(43–49)–hCGβ(106–116)	CMECDI	TT	2	2	2	1	1	1
4	hCGα(43–49)–hCGβ(110–116)	CMECDI	TT	10	10	7	6	0	0
B									
5	hCGα(46–55)–hCGβ(106–116)	Glutaraldehyde	TT	10	10	9	9	0	0
6	hCGα(46–55)–hCGβ(106–116)YG	Benzidine	KLH	10	10	10	10	0	0
7	hCGα(46–55)–hCGβ(106–116)YG	TMB	TT	10	2	2	1	0	0

*A significant response in ELISA was determined as the absorbance (492 nm) value >1 at a serum dilution of 1:1000 after five injections. †A significant response in RIA was determined as the percentage of binding >5% at a serum dilution of 1:100 after five injections.

served with a MAb designated FB12 and directed to the hCGβ(110–116) region (*20*). In contrast, inhibition profiles observed with antiserum R9-4 showed that two different populations of antipeptide antibodies were present in this antiserum, the first directed to the hCGβ portion and the second to an epitope present on both α and β regions, since inhibition of up to 36 and 79%, respectively, was obtained with the free hCGβ subunit and the recombinant hCGα-hCGβ products. Products formed by recombination of hCGα with hCGβ inhibited the binding of ^{125}I-hCG to antiserum R40-7, whereas the free subunits did not, thus the entire population of Abs to the peptide in this antiserum was to a discontinuous antigenic site comprising amino acid residues of both hCGα and hCGβ subunits. Two major epitopes, therefore were present on the hCGα(46–55)–hCGβ(106–116) peptide: a continuous epitope located within the hCGβ portion and a quaternary protein–specific epitope of hCG comprising amino acid residues of both the hCGα(46–55) and the hCGβ(106–116) regions.

We also studied the capacity of antipeptide antibodies elicited by hCGα(46–55)–hCGβ(106–116) peptide-carrier conjugates to inhibit the binding of hCG to the CG-LH receptor (*21*). Radioreceptor assays using ^{125}I-hCG showed that antipeptide antibodies R9-4, R10-4, R37-6, and R40-7 were capable of inhibiting the binding of ^{125}I-hCG to its receptor, as were both hCG and MAb C8 (Fig. 4).

Our results show that the delineation of an antigenic size recognized by a MAb specific for hCG enables the definition of a quaternary protein–specific epitope present on this hormone and the construction of a synthetic immunogen mimicking such an epitope. Peptides can elicit Abs of predetermined specificity, but were described as raising antibodies directed to continuous epitopes. We now show that a synthetic immunogen can elicit Abs to a conformational epitope that are specific for a heterodimeric structure. These experiments were carried out in rabbits with a peptide mimicking an epitope on human CG, thus raising the question as to whether the same species can make antipeptide Abs to its own CG-LH. A peptide spanning the α(50–59)–β(106–116) portions of rat LH (rLH) and corresponding to the hCGα(46–55)–hCGβ(106–116) regions was synthesized and conjugated to TT. In five female rats injected with the peptide-carrier conjugate, Abs to rLH were consistently detected by RIA (*22*), indicating that the synthetic immunogen is effective when used in the same species.

The capacity of a synthetic peptide to mimic a conformational epitope is of particular interest for the development of an hCG vaccine for producing infertility and can be applied not only to the construction of potential vaccines (*17, 23*) but also to the synthesis of immunogens to generate MAb probes for use in other disciplines.

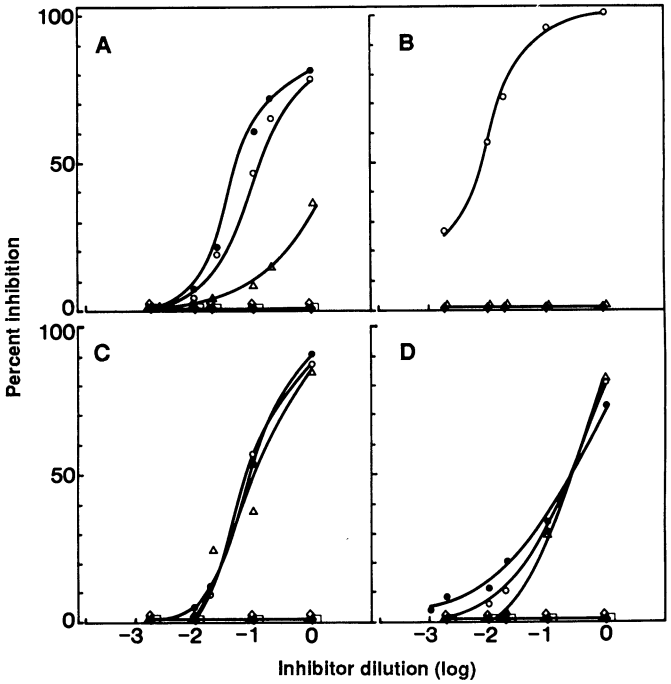

Fig. 3. Characterization of the antigenic site recognized by antiserum (**A**) R9-4, (**B**) R40-7, (**C**) R37-6, or (**D**) R10-4 by competitive inhibition experiments with recombinant products. These products were obtained by recombination of α subunit (4 μg) with β subunit (5 μg) after incubation for 20 hours at 4°C (*9*); they were used to inhibit binding of ^{125}I-hCG to these antisera. Experiments were performed by RIA (*19*), and results are expressed in percent inhibition of ^{125}I-hCG binding. The indicated values represent the arithmetic mean of duplicate samples (as explained in Fig. 1). The SD in all experiments is <10%; and each experiment was reproduced at least twice with similar results. hCGα (◆), hCGβ (△), ovine LHα (◇), hLHβ (▲), hCGα-hCGβ (○), hCGα-hLHβ (□), and ovine LHα–hCGβ (●).

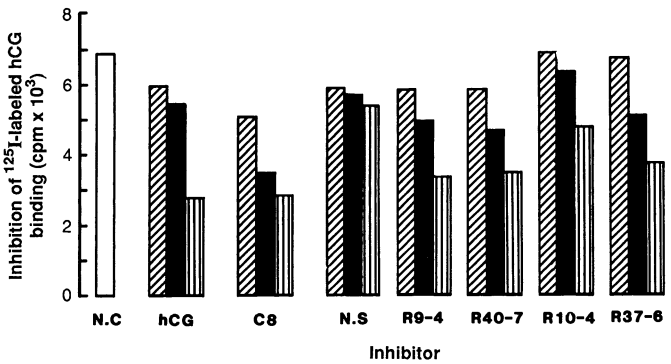

Fig. 4. Inhibition of ^{125}I-hCG binding to the CG-LH receptor by hCG, MAb C8, or antiserum R9-4, R40-7, R10-4, or R37-6. Experiments were performed by radioreceptor assay with inhibitors diluted at 1:400 (bars with diagonal lines), 1:200 (solid bars), and 1:40 (bars with vertical lines) from the initial source of reagent (*21*). NS, nonspecific rabbit antiserum; NC, negative control corresponding to the maximal binding of ^{125}I-hCG to the CG-LH receptor in the absence of an inhibitor. The indicated values (counts per minute) represent the arithmetic mean of duplicate samples (as explained in Fig. 1). The SDs were all less than 10% of the sample mean; and each experiment was reproduced at least twice with similar results.

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10. Briefly, C8 antibody-coated polystyrene plastic beads were incubated with the recombinant products (200 μl) for 2 hours at room temperature. After washing, ^{125}I-labeled FBT 10 (100,000 cpm, 200 μl) was added for a second incubation of 1 hour at room temperature. Then, the beads were washed, and the radioactivity bound was counted. MAb FBT 10 binds free and combined hCGβ as well as hLHβ.
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12. The peptides were synthesized [R. B. Merrifield, *J. Am. Chem. Soc.* **85**, 2149 (1963)] on a model 430A Applied Biosystems solid-phase synthesizer by using two kinds of solid-phase resins: 4-methylbenzhydrylamine for obtaining $CONH_2$ peptides and phenyl-acetamidomethyl resins loaded with *tert*-butyloxycarbonyl amino acids for obtaining COOH peptides. After synthesis, the peptides were depro-

tected and cleaved in two steps from the resin with anhydrous fluorhydric acid. After purification by exclusion chromatography and high-performance liquid chromatography, the purity and identity of each peptide were checked by amino acid analysis and microsequencing.

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14. Seven groups of female rabbits (Grand Fauve de Bourgogne, Centre d'élevage Gontrand Achard de la vente, Orne, France), consisting of two to ten animals each, received primary immunizations of peptide-carrier conjugate emulsified in complete Freund's adjuvant at multiple intradermal sites. All rabbits received 200 μg of equivalent peptide except those injected with a peptide from batch 7, which received only 15 μg of equivalent peptide. Secondary immunizations were performed with the same quantities of immunogen in incomplete Freund's adjuvant injected subcutaneously 10, 20, 30, and 40 days after the first immunization. Sera were collected before immunizations and on days 23, 33, and 43 after the first injection and stored at −20°C until assayed.
15. Peptide-coated microtiter plates were incubated with various dilutions of antisera [P. Motte, G. Alberici, M. Ait-Abdellah, D. Bellet, *J. Immunol.* **138**, 3332 (1987)]. After washing, the plates were incubated with a 1:1000 dilution of peroxidase-conjugated rabbit antibodies to mouse immunoglobulin for 1 hour at 37°C. Finally, enzyme activity was determined with *o*-phenylenediamine as a chromogenic substrate, and absorbance was measured at 492 nm.
16. The iodogen method was used to label hCG, hCGα, and hCGβ (100 μCi/μg; 1 Ci = 3.7×10^{10} Becquerels) [P. J. Fraker and J. C. Speck, *Biochem. Biophys. Res. Commun.* **80**, 849 (1978)]. ^{125}I-labeled hLH was purchased from the Commissariat à l'Energie Atomique, Saclay, France. Radiolabeled hormones or subunits (30,000 cpm) were incubated overnight at 4°C with serial dilutions of antiserum. The antigen-antibody complex was precipitated by polyethylene glycol and, after centrifugation, the radioactivity of the pellet was counted [J. M. Bidart *et al.*, *J. Biol. Chem.* **262**, 8551 (1987)].
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19. Serial dilutions of recombinant products (50 μl) were incubated with the dilution of antiserum (100 μl) which produced 50% binding to ^{125}I-hCG, and with ^{125}I-hCG (30,000 cpm, 50 μl). After an overnight incubation at 4°C, the ^{125}I-hCG–antibody complex was precipitated by polyethylene glycol. After centrifugation, the radioactivity of the pellet was counted.
20. J. M. Bidart, D. H. Bellet, G. F. Alberici, F. Van Besien, C. J. Bohuon, *Mol. Immunol.* **24**, 339 (1987).
21. Experiments were performed with either hCG (Pregnyl, Organon, Serifontaine, 50 IU/ml), C8 ascitic fluid diluted 1:100, or various antisera as initial sources of reagent. These reagents (50 μl) were used at various dilutions 1:40 to 1:400) and were incubated with 50 μl of ^{125}I-hCG (100,000 cpm) for 18 hours at 4°C. Fresh frozen rat testes were homogenized in ice-cold 0.01 M phosphate buffer and 0.14 M NaCl, pH 7.4 (P_i-NaCl) and centrifuged at 5000*g* for 15 min. The pellet was suspended in P_i-NaCl and then added (100 μl) to preincubated samples and agitated continuously for 1 hour at room temperature. After washing, suspensions were centrifugated at 5000*g* for 15 min, and the radioactivity of the pellet was counted.
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24. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

25. The Center for Population Research of the National Institute of Child Health and Human Development and National Institutes of Health are acknowledged for their gifts of purified hCG (CR-125, 11,900 IU/mg), hCGβ (CR-125), hCGα (CR-123) and hLHβ (AFP 3282B) used in this study. We thank H. Papkoff, E. Burzawa-Gerard, and D. N. Ward for their generous gifts of turtle LHα, carp GTHα, and ovine LHα, respectively, and are indebted to F. C. Lafon for his continuous help. Supported by a grant from Association pour la Recherche sur le Cancer, Villejuif, and a grant from Laboratoire L. Lafon, Maisons-Alfort, France.

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Regulation of Alloreactivity in Vivo by a Soluble Form of the Interleukin-1 Receptor

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In vitro studies have shown that cytokines are involved in the regulation of the immune response, but their role in vivo is less well defined. Specific cytokine antagonists enable the identification of particular cytokines involved in the response and offer a means for modifying it. Systemic administration of a soluble, extracellular portion of the receptor for interleukin-1 (sIL-1R) had profound inhibitory effects on the development of in vivo alloreactivity. Survival of heterotopic heart allografts was prolonged from 12 days in controls to 17 days in mice treated with sIL-1R. Lymph node hyperplasia in response to a localized injection of allogeneic cells was completely blocked by sIL-1R treatment. The inhibition was overcome by simultaneous administration of interleukin-1 (IL-1); thus, sIL-1R acts by neutralizing IL-1. These results implicate IL-1 as a regulator of allograft rejection and demonstrate the in vivo biological efficacy of a soluble cytokine receptor.

INTERLEUKIN-1α AND INTERLEUKIN-1β (IL-1α and IL-1β) are cytokines involved in regulation of the immune and the inflammatory response (*1, 2*). Despite their distinct primary amino acid sequence (*3*), both IL-1α and IL-1β bind to the same specific plasma membrane receptor (*4–8*). The IL-1 receptor (IL-1R) on murine T cells was identified by cDNA expression cloning and NH_2-terminal sequence analysis as an integral membrane glycoprotein of M_r 80,000 (*9, 10*). When a cDNA encoding 316 residues of the extracellular region of the murine IL-1R is expressed in HeLa cells, they secrete a soluble IL-1 binding protein into the culture medium (*11*). Although it is not sufficient for signal transduction (*12*), this soluble, truncated receptor (sIL-1R) exhibits IL-1 binding properties similar to those of the full-length receptor (*11*) and, as such, represents a molecule with the potential to inhibit the biological activities of IL-1. To determine if sIL-1R would modulate the immune response in vivo, we measured its effects on the rejection of cardiac allografts and on the in vivo lymphoproliferation that occurs after challenge with allogeneic cells.

Neonatal C57BL/6 (H-2^b major histocompatibility complex haplotype) hearts were transplanted into the ear pinnae of BALB/c (H-2^d) hosts. Survival of the transplanted hearts was assessed by visual inspection of the grafts for pulsatile activity. Such grafts survived 11 to 13 days in individual control mice treated with mouse serum albumin (MSA) (Table 1). When allograft recipients were given three to six daily injections of sIL-1R, graft survival was prolonged in every case. The mean graft survival time in sIL-1R–treated mice (17 to 18 days) was 5 to 6 days longer than the survival time of identical grafts in control mice (Table 1). The prolongation of cardiac allograft survival achieved with this short-course treatment of sIL-1R is similar to or greater than that observed in a similar grafting system with other immunosuppressive regimens, such as treatment with antibodies to CD4 glycoprotein or total lymphoid irradiation (*13*). Prolongation of graft survival with sIL-1R was not as great as that observed after treatment with antibody to the IL-2 receptor in a different grafting model (*14, 15*). A direct comparison of these various immunosuppressive agents would be necessary to determine their relative efficacies.

The effect of sIL-1R on alloreactivity in vivo was also examined in a second experimental system, involving the localized, T cell–dependent, lymphoproliferative response to allogeneic cells. BALB/c mice were exposed to irradiated, allogeneic

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