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Conjugation of BbCG to the carrier

Derivatization and activation of carrier:

Tetanus toxoid (5000 Lf/mg protein nitrogen, 2000 Lf/ml) and diphtheria toxoid (6000 Lf/mg protein nitrogen, 2000 Lf/ml) were obtained from Serum Institute, Pune, India, and were derivatized using the hetero bifunctional reagent N-Succinimidyl-3-(2-Pyridyldithio) Propionate (SPDP), (Pharmacia, Sweden). The protein, dissolved in 0.1 M phosphate buffer with 0.1 M NaCl, pH 7.5 (PBS), was treated with SPDP dissolved in absolute ethanol to achieve different protein:SPDP ratios. The reaction was carried out at 25°C for 30 min with constant stirring. Free SPDP was then removed by passing the mixture through a Sephadex G-25 column (1x30 cm), equilibrated with PBS containing 5 mM EDTA, pH 6.8. The fractions corresponding to the protein peak were pooled and concentrated in an Amicon ultrafiltration assembly. The extent of derivatization was evaluated as follows. Briefly, 100 μl of the modified carriers were diluted to 1 ml with 0.1 M dithiothreitol (DTT, Sigma Chemical Co., USA) in PBS, pH 7.5 incubated for 10 min at 25°C. The absorbance of the released 2-pyridylthione was measured at 343 nM and the extent of activation calculated using the absorption coefficient of 2-pyridylthione (E_{343} 8080) (Carlsson et al 1978). Activation of these carriers was achieved by adding DTT to a final concentration of 50 mM. After
incubating for 15 min at 25°C, the reaction mixture was applied to a Sephadex G-25 column (1 x30 cm). The elution buffer was saturated with nitrogen before use.

**Activation of βhCG**

β subunit of hCG was treated with a five molar excess of SMCC (Succinimidyl 4 (N-Maleimido methyl) Cyclohexane-1-Carboxylate, Pierce Chem. Co.). The reaction was carried out in PBS for 45 min at room temperature (RT) with constant shaking. The removal of free unreacted SMCC was accomplished by elution through a Sephadex G-25 column.

**Conjugation of β-hCG with the carrier**

SPDP-activated carriers (tetanus toxoid or diphtheria toxoid) and β-hCG were mixed in different molar ratios (1:1, 1:5, 1:10, 1:15, 1:20) in nitrogen-flushed glass containers and are allowed to react for 18 hours at 4°C. The conjugates were purified on HPLC using an LKB TSK G-3000 SW column (7.5x60 cm) and dialyzed extensively against normal saline, filter sterilized through a 0.22 μm filter and stored in a steril nitrogen-flushed container. The gonadotropin content in the conjugates was estimated by the phenol-sulphuric acid method using β-hCG as a standard.


**Analysis of the conjugates**

**Electrophoresis**

The conjugates were analysed by agarose gel electrophoresis as the electrophoretic mobility of these toxoid-carrier conjugates were poor in polyacrylamide gels. 1 mm thick 1% agarose gels in barbitone buffer pH 8.6. were used for electrophoresis. 20 μg of the conjugates were applied per lane and run at a constant current of 5 mA per lane. The gels were stained with Coomassie brilliant blue R-250.

**Estimation of βhCG in the conjugates**

**Radio immunoassay :**

The βhCG content in the conjugates was estimated by using a βhCG-specific monoclonal antibody in a cold displacement radioimmunoassay. The ascites of the βhCG-specific monoclonal antibody was titrated by dilution to achieve about 20%-30% binding of the $^{125}$I-hCG. βhCG content in conjugates was measured as follows; 50 μl of the diluted conjugate sample or standard hCG, 50 μl of normal horse serum (diluted 1:2.5 in assay buffer), 50 μl of appropriately diluted monoclonal antibody, 50μl of $^{125}$I-hCG (10,000 cpm), were incubated for 18 hr at 4°C. The immune complexes were precipitated using 1 ml of 66% ammonium acetate-alcohol, centrifuged and counted in a LKB gamma-counter. The values of βhCG were calculated from the standard curve.
Carbohydrate estimation:

βhCG content in the conjugates was also estimated in terms of the carbohydrate content present in the molecule. The carbohydrate in the conjugates were measured using phenol-sulphuric acid method (Dubois et al 1956) taking βhCG as standard. Briefly, to 200 µl of sample or standard, 200 µl of 5% phenol and 1 ml of concentrated sulphuric acid were added while stirring, cooled and the absorbance read at 490 nm.

Conjugation of αoLH-βhCG to TT

Formation of heterospecies dimer (HSD)

Purified β subunit of hCG (13,000 IU/mg) was annealed to αoLH by following essentially the procedure described by Aloj et al (1978). Molar equivalents of βhCG and αoLH were mixed in phosphate buffer (25 mM, pH 7.0, at 2.5 mg/ml protein concentration) at 37°C for 18 hr. The association of the heterospecies dimer (HSD) was confirmed in a Leydig cell bioassay to assess its ability to stimulate testosterone.

Bioassay of the heterospecies dimer

All glasswares were siliconized before use. 8-12 weeks old adult NMRI mice were sacrificed by cervical dislocation, their testes removed and minced in cold DMEM supplemented with 2 mM glutamine and 2% FCS. The
homogenate was filtered through a nylon mesh and gently stirred at 4°C for 5 min to assist the separation of individual cells. Cells were then incubated at 34°C for one hour in a 5% CO₂ atmosphere and centrifuged at 400xg. The supernatant was discarded and cells resuspended in 10 ml of fresh medium per pair of testes. 200 μl of this suspension was aliquoted into tubes containing standard hCG (10,000 IU/mg) in the range of 0 to 50 pg or the test αoLH-BhCG in sodium-potassium phosphate 10 mM, pH 7.4, containing 0.14 M NaCl, 0.1% BSA, 50 μg/ml streptomycin, and 50 IU/ml penicillin. Incubations were carried out for three hours under the conditions mentioned above, after which cells were killed by heating at 70°C for 15 min. The testosterone produced was estimated by radioimmunoassay using WHO matched reagents as described in the WHO Radioimmunoassay Protocol Handbook (1989).

Duplicate samples containing testosterone were extracted with 1.5 ml of ether in duplicate. The ether layer was transferred to separate tubes after freezing the aqueous layer in dry ice and evaporated to dryness at room temperature. 500 μl of the assay buffer (PBS, 0.1 M, pH 7.2 containing 0.1 M NaCl and 0.1% gelatin) was added and vortexed. 100 μl of antiserum diluted appropriately, and 100 μl of the working dilution of ³H-testosterone (100 nCi/ml) were added and incubated at 4°C for 24 hr. To remove the free from the antibody-bound hormone 200 μl of dextran-charcoal (0.625 g charcoal, 0.0625 g dextran in
100 ml assay buffer) was added, vortexed and incubated for 45 min. The tubes were centrifuged at 1000xg for 5 min, and the supernatants decanted immediately into scintillation vials. Scintillation cocktail was added and the samples counted in an LKB beta counter. Testosterone levels in the samples were estimated by comparing the samples with known standards.

**Conjugation of the aoLH-βhCG dimer to TT**

aoLH and βhCG when mixed in solution are known to anneal irreversibly (Aloj et al 1978). 15 mg of annealed aoLH-βhCG in 0.1 M phosphate buffer, pH 7.5, was activated for 30 minutes, at 25 °C, with a ten molar excess of succinimidyl 4- (N-maleimidomethyl cyclohexane-1-carboxylate (SMCC) (Pierce Chemical Company, USA). Excess unreacted SMCC was separated by column chromatography on Sephadex G-25 (LKB-Pharmacia, Sweden). This mixture was passed through a Sephadex G-25 column and eluted with 0.1 M phosphate buffer, pH 7.5. Fractions corresponding to the aoLH-βhCG peak were pooled. Tetanus toxoid (or TT peptide) was activated for 30 minutes, at 25°C, with a 100 molar excess of N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Pierce Chemical Company, USA). Excess unreacted SPDP was separated by column chromatography on Sephadex G-25. SPDP-activated TT was then reduced with DTT (Pierce Chemical Company, U.S.A.) for 30 minutes at 25°C and then desalted on a Sephadex...
G-25 column. Activated αoLH-BhCG and TT were mixed and incubated at 25°C for 2 hours and then at 4°C for 18 hours. The entire procedure was carried out in an atmosphere of nitrogen, using nitrogen-flushed tubes and buffers. The conjugates were dialyzed extensively against 10 mM PBS, pH 7.4 and then analyzed by HPLC using a TSK G3000 SW (LKB-Pharmacia, Sweden) column.
**Immunogenicity testing of conjugates with different stoichiometries**

**Immunization:**

Adult BALB/c mice were used for the immunogenicity studies. Each experimental group consisted of ten adult mice, which were injected thrice at monthly intervals with 100 μl of the conjugate containing 10 μg gonadotropin. All the injections were given using aluminum hydroxide (alum) as the adjuvant. Mice were bled on day 67 and assayed for anti-hCG and anti-carrier (TT or DT) antibodies.

**Assay for anti-hCG antibodies**

Anti-hCG antibodies were estimated by a liquid-phase radioimmunoassay (RIA) using ¹²⁵I-labeled hCG as described by Rothbard et al. Briefly, 50 μl of appropriately diluted test serum, 50 μl of normal horse serum (added as carrier protein), 50 μl of ¹²⁵I-hCG (10,000 cpm per tube) and 50 μl of a predetermined amount of unlabeled hCG in assay buffer (PBS, pH 7.4, containing 0.1% BSA) were incubated at 4°C for 18 hours and precipitated with ammonium-acetate alcohol. The tubes were centrifuged and the radioactivity in the pellets estimated in a LKB gamma counter (LKB-Pharmacia, Sweden). Antigen-binding capacity of each serum was calculated by non-linear least square analysis.
Assay for anti-TT and DT antibodies

Anti-TT and DT antibodies were estimated by ELISA. 100 μl volumes of TT or DT at a concentration of 10 μg/ml in carbonate buffer (50 mM, pH 9.5) were coated onto 96 well ELISA plates (Nunc, Denmark) by overnight incubation at 4°C. The plates were washed with PBS containing 0.2% Tween-20. 100 μl of sera in doubling dilutions starting from 1:200 in PBS-Tween were added to the antigen-coated wells and the plates incubated for 1 hour at 37°C. After another wash with PBS-Tween, anti-TT antibodies were revealed by anti-mouse immunoglobulin conjugated to horse radish peroxidase and incubated for 1 hour at 37°C. Plates were washed with PBS-Tween followed by the addition of 100 μl per well of substrate [0.15 M citrate phosphate buffer, pH 5.0, containing 0.5 mg per ml of o-phenylene diamine (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 0.03% hydrogen peroxide]. The plates were incubated in the dark for 20 minutes. Further development of colour was arrested by the addition of 50 μl of 5N sulfuric acid and the absorbance measured at 490 nM in an ELISA Reader (Molecular Devices Corporation, U.S.A.). Antibody units were calculated as the mean of the product of the absorbance and the corresponding dilution in the linear range.
Effect of carrier-presensitization on anti-ligand responses

Immunization protocol in the BhCG-TT system

To simulate carrier-induced epitope specific suppression in animals, two groups of BALB/c adult mice (10 animals per group) were presensitized with TT adsorbed on alum. On day 0, one group received 10 µg and the other 100 µg of TT, whereas the control group received alum and saline alone. This was followed by intramuscular injections of 10 µg equivalent of gonadotropin of the BhCG-TT conjugate on days 30, 60 and 90. The mice were bleed on days 37, 67 and 97 and analysed for the presence of anti-BhCG and anti-TT antibodies as described earlier.

Bypass of carrier induced suppression by the use of an alternate carrier

Animals from the group presensitized with 100 µg TT were later injected with (10 µg BhCG equivalent) BhCG linked to DT adsorbed on alum on day 120 and 150. The bleeds were collected on days 127 and 157, and assayed for anti-hCG, anti-TT and anti-DT antibodies.

Immunization protocols in the αoLH-BhCG-TT system

Six to eight week old C3H/He (H-2^K), BALB/c (H-2^d), SJL (H-2^s) and C57BL/6 (H-2^b) (Jackson Laboratories, USA)
bred in our animal house were used in these experiments. Groups of eight mice each were presensitized with 100 μg of TT, absorbed on alum, by the intraperitoneal route. Mice in the control group received only alum and saline. Thirty days later, control and experimental animals were immunized intramuscularly with a 10 μg equivalent of oLH-hCG conjugated to TT absorbed on alum. This was followed by booster immunizations with the same preparation 60 and 90 days after the initial presensitization dose of TT. Animals were bled on days 67 and 97 and the titres of anti-hCG, anti-oLH and anti-TT antibodies estimated.

Assay for anti-oLH antibodies

Anti-oLH antibody titres were estimated by an indirect ELISA. 1 μg per well of oLH in carbonate-bicarbonate buffer, pH 9.6, was coated onto 96 well plates. This was followed by incubation of the test sera at serial dilutions starting from 1:200, and subsequent washing with PBS-Tween. Sheep anti-mouse immunoglobulin antibodies (WHO Reference Reagent Center, Birmingham, U.K.) at a dilution of 1:1000 was added and the plates incubated for 1 hour. After another washing step, HRP conjugated anti-sheep immunoglobulins were added as revealing antibodies and the plates incubated for a further period of 1 hour. Plates were washed with PBS-Tween followed by the addition of 100 μl per well of
substrate [0.15 M citrate phosphate buffer, pH 5.0, containing 0.5 mg per ml of o-phenylene diamine (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 0.03% hydrogen peroxide]. The plates were incubated in the dark for 20 minutes. Further development of color was arrested by the addition of 50 μl of 5 N sulphuric acid and the absorbance measured at 490 nM in an ELISA Reader (Molecular Devices Corporation, U.S.A.). Antibody units were calculated as the mean of the product of the absorbance and the corresponding dilution in the linear range.

**Estimation of IgG subclasses**

1 μg per well of the appropriate antigen (βhCG or αoLH) was coated onto microtitre plates as described above and the sera at a dilution of 1:1000, was dispensed onto the antigen-coated wells followed by incubation at 37°C for 1 hour. This was followed by the addition of an appropriate dilution of subclass-specific sheep anti-mouse antibodies (WHO Reference Reagent Center, Birmingham, U.K.). After washing the plates with PBS-Tween, HRP-conjugated anti-sheep Ig antibody was added and the plates incubated at 37°C for 1 hour. Colour development was done as described above.
Bypass of carrier-induced suppression by the use of a synthetic Th epitope

Synthesis of peptides

Synthesis of peptides was done by a stepwise solid phase procedure using the Model 430A peptide synthesizer (Applied Biosystems, Foster City, CA, U.S.A.). Synthesis was carried out on a 0.5 mmol scale using PAM resin. The couplings were done with preformed Boc-amino acid symmetric anhydrides, with the exception of glutamine and asparagine which were coupled as hydroxybenzotriazole esters. The following side-chain protecting groups were used: Asp (Bzl), Ser (Bzl), Tyr (Br-z) and Lys (Cl-z). Peptides were deprotected and cleaved from resin using trifluoromethanesulfonic acid and trifluoroacetic acid-thioanisole (Fluka) (Tam and Merrifield 1987) and then purified by gel filtration on Sephadex G-15 (LKB-Pharmacia, Sweden) in 10% acetic acid followed by high pressure liquid chromatography on a C-18 column (Applied Biosystems, Foster City, CA, U.S.A.). Elution was performed using the following protocol: Solvent A - 0.05% trifluoroacetic acid in water, Solvent B - 0.05% trifluoroacetic acid in acetonitrile, linear gradient of 5-95% Solvent B in 40 minutes at a flow rate of 1.5 ml/min; detection at UV 226 nm.

Purity of the peptides was assessed by analytical reverse phase HPLC and further purifications were carried out if
necessary, on a C-18 column. The amino acid compositions of these synthetic peptides were confirmed by amino acid analysis.

**Amino Acid Analysis**

20-30 μg of the purified peptide was hydrolyzed using concentrated HCl at 120°C for 18 hr. in sealed tubes. The excess HCl was neutralized with ethanol/water/triethylamine (2:2:1), vortexed and evaporated. The amino acids thus obtained were derivatized using ethanol/water/triethylamine/PITC (7:1:1:1) for 15 min at room temperature, vortexed and the excess liquid evaporated. The sample was solubilized and diluted in 5 mM phosphate buffer pH 7.4, containing 5% acetonitrile. The mixture of derivatized amino acids were analyzed in a Waters amino acid analyzer using a PICO Tag column, and a gradient of sodium acetate/triethyl amine/acetic acid buffer pH 6.4 containing 6% acetonitrile (solvent A) and 60% acetonitrile 40% water (solvent B). The gradient was formed between 0% B to 100% B in 10.5 min at a flow rate of 1 ml/min. The separated derivatized amino acids were detected at 254 nM.

**Antigen-induced cell proliferation assays**

C57BL/6, BALB/c, SJL and C3H/He mice were immunized in the footpads with either 10 μg of tetanus toxoid or 100 μg of peptide emulsified in complete Freund's adjuvant
(CFA). Eight days later the animals were sacrificed and the inguinal and popliteal lymph nodes removed under aseptic conditions. Single cell suspensions were made by gently teasing the lymph nodes and running the suspensions through nylon mesh. Cells were cultured in 96 well plates (Nunc, Denmark) at a concentration of $5 \times 10^5$ cells per well in RPMI-1640 (GIBCO, NY, USA) supplemented with 0.5% normal mouse serum, 2.5 mM glutamine and 50 $\mu$M mercaptoethanol. Peptides and TT were added to the wells at various concentrations (ranging from 0.1 $\mu$g to 100 $\mu$g) in a volume of 100 $\mu$l of medium. This was followed by incubation at 37°C, at 5% CO$_2$ for a period of 72 hours. At the end of this period, 1 $\mu$Ci of $^3$H-thymidine (New England Nuclear, Boston, MA, USA) was added to each of the wells and the incubation continued for another 18 hours. Cell proliferation was estimated by evaluating the incorporation of radioactive thymidine.

**Conjugation of aoLH-BhCG to synthetic Th epitope**

The aoLH-BhCG (HSD) was activated with SMCC as previously mentioned in aoLH-BhCG-tetanus toxoid conjugation. The peptide containing cysteine was treated with 0.25M dithiotheritol at 40°C for 20 min. and then passed through Sephadex G-25 (1x30) eluted with phosphate buffer 0.1 M containing 0.1 M NaCl and 5mM EDTA (degassed and flushed with nitrogen). The activated aoLH-BhCG and peptide were mixed and left at 4°C in nitrogen flushed container for
18 hr. The mixture was dialyzed against normal saline, filter-sterilized and stored at 4°C till used.

**Immunization protocol**

Mice were presensitized (SJL H-2<sup>S</sup>, eight animals/group) by injecting with 100 μg of tetanus toxoid adsorbed on alum on day 0. Control groups received alum and saline alone. This was followed by injections of 10 μg/animal gonadotropin equivalent of the conjugate (αoLH-βhCG-TT or αoLH-βhCG-peptide) on day 30, 60, 90 (Table I). Animals were bled on days 67 and 97 and analyzed for the antibody titres.

**Antibodies to hCG, TT, DT, αoLH, IgG Subclasses**

The antibodies to hCG, TT and DT were estimated as described earlier in "Immunogenicity testing of conjugates". Anti αoLH and IgG subclasses were measured as described in "Effect of carrier-presensitization".