MATERIALS & METHODS
2.1 MATERIALS:

2.1.1 Hormones, Vaccine and peptides: Highly pure hCG (10,000 IU/mg), αhCG and βhCG were provided by Mr. P. Sahai, GMP/GLP unit, NII. HLH was a kind gift of Dr. Brij Saxena, Cornell University, USA. I25I-hLH and I25I-hFSH were obtained from the W.H.O. Matched RIA Reagent Program, Geneva. I25I-hCG was kindly provided by Dr. Om Singh, Immunoendocrinology lab., NII. The β-hCG peptides 38-57 and 109-145 were a kind gift by Dr. V. C. Stevens, Ohio State University, Columbus, USA. The βhCG peptide 109-118 was a kind gift of Dr. K. Iyer, IRR, Bombay. HSD-TT, HSD-DT and DT were given by Mr. P. Sahai, GMP/GLP unit NII. The HSD vaccine adsorbed on to alum was provided by Dr. Rahul Pal, Immunoendocrinology lab, NII.

2.1.2 Tissue Culture: All sterile plastic ware used for tissue culture was obtained from Corning, USA. RPMI-1640, HEPES, HAT, sodium bicarbonate, polyethylene glycol (PEG) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co., USA. Fetal calf serum was obtained from Hyclone Lab Inc., USA.

2.1.3 Reagents for Immunoassays: Microtitration plates were from Titertek, USA and Corning, USA. Bovine serum albumin (BSA), Tween-20, PEG-8000, Trizma base were purchased from Sigma Chemical Co., USA. The o-phenylenediamine was from USB, USA.

2.1.4 Chemicals for Electrophoresis and Western blotting: Tris, glycine, acrylamide, N'N' methylene bisacrylamide, sodium dodecyl sulphate (SDS), TEMED, ammonium persulphate and prestained molecular weight standards were obtained from Bethesda Research Laboratories, USA. Nitrocellulose paper (0.45µm) was from Amersham.
and α-chloronaphthol was from USB, USA.

2.1.5 Other reagents: Freund's complete adjuvant, Freund's incomplete adjuvant, Pristane, horseradish peroxidase, sodium borohydride, glycerol and dithiothreitol (DTT) were purchased from Sigma Chemical Co., USA. Iodoacetamide was obtained from USB, USA.

2.1.6 Antibodies: Monoclonal antibody 357-2 which recognizes βhCG loop peptide 38-57 and rabbit anti-CTP antisera were kindly provided by Dr. V. C. Stevens, University of Ohio, USA. Affinity purified, peroxidase conjugated rabbit anti-mouse Ig and rabbit anti-human Ig were purchased from Dakopatts, USA. Peroxidase conjugated Protein-A, Peroxidase conjugated goat anti-mouse IgG (Fc), human IgG standards and goat anti-human IgG (F(ab)2) were from Sigma Chemical Co., USA. Goat antimouse Ig, goat anti-rat IgG and mouse IgG standards were obtained from NII Reagent Bank. Mouse monoclonal antibodies to human IgG1 (HP6012), IgG3 (HP6050) and IgG4 (HP6101) were purchased from Zymed Lab. Inc., USA and to human IgG2 (HP6014) was from Sigma Chemical Co., USA. Human IgG subclass standards were purchased from Sigma Chemical Co., USA.

METHODS:

2.2 GENERATION OF MONOCLONAL ANTIBODIES:

Anti-hCG monoclonal antibodies were generated using protocols standardized in the lab (Gupta and Talwar, 1983), the details of which are given below.

2.2.1 Immunization Schedule: Three groups of female BALB/c mice (8 animals/group) reared in the Institute's Small Animal Facility were immunized subcutaneously with 10µg of hCG, β-hCG or hLH per animal
in Freund's complete adjuvant respectively. Animals were boosted after four weeks with 10μg of respective antigens intraperitoneally (IP) in Freund's incomplete adjuvant. On days 3, 2 and 1, prior to fusion, animals were given 50μg of antigen in saline, IP.

2.2.2 Myeloma Cells and Immune Splenocytes: X63Ag8.653 myeloma cells were kept in logarithmic phase of growth for at least 4 days prior to fusion by subculturing daily. Prior to fusion, cells were harvested and centrifuged at 500Xg for 10 minutes. Cells were washed twice in plain RPMI-1640 medium and were resuspended in the same medium and counted in a haemocytometer.

After the last booster mice were killed by ether anesthesia and spleens removed aseptically in RPMI-1640 medium. Single cell suspension was made by teasing the spleen with a serrated tooth forceps. Clumps and membrane fragments were allowed to settle and the supernatant was centrifuged at 500Xg for 10 min. Cells were washed twice in RPMI-1640, resuspended and counted in a haemocytometer.

2.2.3 Fusion: Immune splenocytes (1 X 10^7) were mixed with myeloma cells (5 X 10^6) in a 10ml sterile tube, centrifuged at 500Xg for 10 min and washed twice with RPMI-1640 medium. To the pellet, 0.5ml solution of 50% PEG 1300 was added drop by drop with constant stirring. The pellet was rocked continuously for 1 min and 4ml of RPMI-1640 medium was added dropwise. The suspension was mixed intermittently for 4 to 5 min and centrifuged at 500Xg for 10 min. The resulting pellet was resuspended in 10ml of RPMI-1640 containing 20% FCS and HAT. Fused cells (100μl/well) were overlaid on to 24 well culture plates preincubated with 10^5 feeder cells (peritoneal macrophages
from BALB/c mice) per well. The medium was replaced with fresh HAT selection medium on days 3 and 6. Cultures were screened microscopically for the presence of hybrids. On days 14 or 15 supernatants from wells showing hybrids were tested for anti-hCG antibodies by RIA or EIA as described later.

Positive hybrids were further cloned by the technique of limiting dilution in 96 well culture plates so as to get 0.5 to 1 cell per well. Each well was seeded earlier with approximately 1 X 10^4 peritoneal macrophages as feeder cells. One week after plating, wells showing single colonies were screened for the production of anti-hCG MAbs. Positive clones were further subcloned into 96 well plate, till the hybrid cell clones became stable.

2.2.4 Freezing of Hybrid Cell Clones: Hybrid cell clones producing anti-hCG MAbs were frozen for preservation. Cells were grown in culture flasks and harvested in the logarithmic phase of growth. Cells were centrifuged at 800X g for 10 min and resuspended in 10% DMSO in FCS at a concentration of 1 to 2 million cells per ml. One ml of cell suspension was distributed in freezing vials. The vials were closed tightly and transferred to -70°C freezer. After 2-3 days vials were kept in liquid nitrogen till use.

2.3 MONOCLONAL ANTIBODY SUPERNATANT AND ASCITES PRODUCTION: Selected hybridomas were grown in large scale either in tissue culture or as ascites to produce sufficient amount of MAbs.

2.3.1 Production of MAb Supernatant: Hybrid cells were grown in 175-cm2 tissue culture flask containing RPMI-1640 medium with 10% FCS. Cells were split 24 hours later 1:10 in a new 175-cm^2 flask. The
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flask was filled with 50ml of medium and placed in humidified 5% CO₂ incubator at 37°C, until cells were overgrown and medium became acidic (yellow). Tissue culture supernatant was collected into sterile 50ml conical centrifuge tubes and centrifuged at 1800Xg for 10min at room temperature. The resulting supernatant was aliquoted and stored at -20°C till further use.

2.3.2 Production of Ascites: Hybrid cell clones were grown as ascites in the intraperitoneal cavity of inbred strain of BALB/c mice. Mice were sensitized by IP injection of 0.5ml of Pristane. After 10 to 12 days, 10 million healthy hybrid cells were injected IP. Ascites developed after 1-2 week was tapped using an 18G needle and made cell free by centrifuging at 10,000Xg for 20min at 4°C. Supernatant was removed, aliquoted and stored at -20°C till further use.

2.4 PURIFICATION OF MONOCLONAL ANTIBODIES:
Ascites fluid of selected MAbs was given an ammonium sulfate cut as described by Arunan (1992). Cold saturated ammonium sulfate was added drop by drop to ascites fluid with constant mixing, at a final concentration of 45% (vol/vol). The mixture was stirred for 2-4 hr at 4°C and then centrifuged at 10,000Xg for 15min. This procedure was repeated once more. The pellet was dissolved in minimum volume of PBS and dialyzed against PBS for 24 to 48 hr at 4°C with 3 changes of PBS. After dialysis antibodies were further purified on a protein G column using manufacturers instructions. Briefly, Protein-G column was equilibrated with 30mM of phosphate buffered saline, pH 7.4. The column was loaded with 1ml of protein solution and washed extensively with the same buffer. A flow rate of 30ml/hr was maintained and 2 ml fractions were collected.
Absorbance was monitored at 280nm. Bound IgG antibodies were eluted using 100mM glycine-HCl buffer, pH 2.5. All fractions were immediately neutralized using 1M Tris. Purity of antibodies was checked in SDS-PAGE and pure fractions were pooled and dialyzed against 10mM phosphate buffer, pH 7.4. After dialysis, antibody solution was concentrated by ultrafiltration in an Amicon assembly using PM-30 membranes. IgG concentration was determined by taking O.D. at 280nm. Aliquots of purified antibody were stored at -20°C till use.

2.5 LABELING OF MONOCLONAL ANTIBODIES:
Protein G purified MAbs were labeled with enzyme horseradish peroxidase by the periodate oxidation method as described previously (Wilson and Nakane, 1978). The antibody enzyme conjugates were dialyzed extensively against PBS, mixed with equal volume of glycerol and stored at -20°C.

2.6 CHARACTERIZATION OF MONOCLONAL ANTIBODIES:
2.6.1 Reactivity with hCG, αhCG, βhCG, hLH and hFSH: Reactivity of MAbs with hCG, hLH and hFSH was determined in direct binding radioimmunoassay as described by Dubey et al., (1976) with few modifications. To 50μl of hybridoma culture soup was added 50μl of iodinated hormone (10,000-20,000 CPM), 50μl of 40% NHS and 50μl of assay buffer (10mM sodium phosphate, 150mM sodium chloride, 0.1% BSA, 0.1% sodium azide, pH 7.4). Tubes were vortexed and kept at 4°C at least for 16hr. After incubation, 200μl of 25% PEG-8000 was added, tubes vortexed and kept at 4°C for 30min. The tubes were centrifuged at 2500Xg for 15min at 4°C and supernatant discarded. Tubes were wiped with tissue paper wicks without touching the pellet and bound
radioactivity counted in a gamma counter. Percentage binding of iodinated hormone with respective MAbs was determined taking the binding with polyclonal sera against respective hormones as 100%. Reactivity of MAbs with hCG and its subunits was determined in an indirect RIA as described earlier (Gupta and Talwar, 1982). Each MAb was titrated against $^{125}$I-hCG to determine the dilution of MAb giving 40 to 50% binding. Increasing amount of unlabeled hormones (100μl) were incubated with appropriate dilution of MAb (100μl), $^{125}$I-hCG (20,000 - 30,000 CPM, 100μl), 20% NHS (100μl) and 100μl assay buffer. Tubes were incubated for 16hr at 4°C. Immune complexes were precipitated by adding 500μl of 25% PEG and counts taken as described above. Concentration of cold hormone giving 50% inhibition in the binding of MAb with $^{125}$I-hCG was denoted as ED$_{50}$.

2.6.2 Determination of Affinity Constants: Affinity of MAbs for hCG was determined in a competitive RIA as described elsewhere (Gupta and Talwar, 1982). Increasing amounts of hCG was used to displace suboptimum amount of MAb from binding to $^{125}$I-hCG. Association constants (Ka) of hCG-antibody interaction were computed by non-linear regression analysis using a computer program, LIGAND (Manson and Rodbard, 1980) on Apple IIe.

2.6.3 Distribution of MAbs Epitopes on hCG: The relative orientation of epitopes recognized by MAbs on hCG was determined in a sandwich enzyme immunoassay as described by Gupta and Talwar (1985a). All additions were of 100μl/well and incubations were done at 37°C for 1hr unless mentioned otherwise. Microtitration plates were coated with Protein G purified MAbs at a concentration of 1μg/well in PBS (50mM
sodium phosphate, 150mM NaCl, pH 7.4). The plates were incubated at 37°C for 1 hr and then at 4°C, overnight. After washing twice with PBS, remaining sites were saturated by incubation with 200μl/well of 1% BSA in PBS and plates were washed thrice with PBS containing 0.05% Tween 20 (PBST). Working dilution of hCG (1μg/ml) made in PBST containing 0.25% BSA was added to each well and plates were further incubated. After three washes with PBST, peroxidase labeled anti-hCG MAbs at an appropriate dilution were added. After incubation and washing 5 times with PBST, color was developed using 0.05% o-phenylenediamine in 50mM citrate phosphate buffer, pH 5.0 with 0.06% H₂O₂. The reaction was stopped after 15min by adding 50μl of 5N H₂SO₄ and absorbance read at 490nm.

2.6.4 Estimation of MAbs Content in Hybridoma Culture Supernatants: MAbs were produced in tissue culture as described above. The concentration of MAbs in the culture soups was determined using a standard capture ELISA as described elsewhere (Brooks et al., 1992). Briefly, microtitration plates were coated with goat anti mouse IgG (500ng/well). Tissue culture supernatants were diluted in PBST containing 0.25% BSA. Standard curve was constructed using mouse IgG standards (1 to 30ng/ml). Bound mouse antibodies were detected using peroxidase labeled goat anti mouse Ig.

2.6.5 Isotyping of MAbs: Isotypes of these antibodies were determined using isotyping kit (Sigma Chemical Co., St. Louis, USA) as per manufacturers instructions.

2.6.6 Inhibition of hCG-receptor Interaction: The ability of MAbs to inhibit the binding of hCG to its receptor was determined in a
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radioreceptor assay as described by Pal et al. (1990). Testes from adult Wistar rats were homogenized in ice cold Tris-HCl buffer, pH 7.4 (50mM Tris, 5mM magnesium chloride, 0.1% bovine serum albumin, 0.1% sodium azide). The homogenate was filtered through cheese cloth and centrifuged at 500Xg for 15 minutes and the resulting pellet resuspended in 8ml of Tris-HCl buffer per pair of testes. Hybridoma culture supernatants were diluted in the Tris-HCl buffer. To 50μl of receptor preparation was added 50μl of 125I-hCG (30,000 - 50,000 CPM) and 100μl of antibody dilutions in duplicates. In control tubes instead of antibody, 100μl of Tris-HCl buffer was added. Non specific binding was determined by adding 100μl of excess cold hCG. Tubes were incubated at 37°C for 2 hours. Two ml of Tris-HCl buffer was added, tubes vortexed and centrifuged at 3000Xg for 15min at 4°C. Radioactivity in the pellet was counted in a gamma counter and percent inhibition calculated.

2.6.7 In Vivo Bioneutralization Capacity of MAbs: The ability of MAbs to neutralize hCG in vivo was tested in an uterine weight gain assay as described previously (Gupta and Talwar, 1982). Prepubertal female BALB/c mice aged 20-21 days were given subcutaneously a total dose of 0.5 IU of hCG dissolved in 0.3 ml of isotonic saline with 0.1% BSA in three equally divided doses. A total of 10μg of monoclonal antibody as hybridoma culture supernatant was given over 3 equally divided daily doses. Control animals received either hCG followed by saline (+ve control) or only saline (-ve controls). Each group had 5 to 6 animals. On day 4 all animals were sacrificed, their uteres removed, blotted and weighed on a microbalance.

2.6.8 Binding of MAbs to hCG-receptor Complex: MAb 206 was
labeled with $^{125}$I by Iodogen method as described elsewhere (Fraker and Speck, 1978). The receptor preparation (250µl) was incubated with 100ng of hCG at 37°C for 1h. Free hCG was separated from hCG-receptor complex by using 3 cycles of centrifugation and washing of the complex with Tris-HCl buffer. MAb 206 (100,000 CPM) was added to the complex and tubes kept overnight at 4°C. Unbound MAb 206 was removed by using 3 cycles of centrifugation and washing and radioactivity counted in a gamma counter. Non specific binding to the receptor was determined in tubes where no hCG was added. The ability of cold antibodies to inhibit the binding of $^{125}$I-MAb 206 with hCG-receptor complex was determined by preincubating hCG-receptor complexes with excess amount of respective antibodies.

2.6.9 Binding of MAbs with HSD Vaccine: The ability of MAbs to recognize HSD-TT and HSD-DT vaccine was determined in a direct binding ELISA. Microtitration plates were coated with respective vaccines at a concentration of 1µg/well. Monoclonal antibodies were used at saturating amounts. Bound anti-hCG MAbs were detected using appropriate dilution of goat anti-mouse IgG-Fc conjugated to horse raddish peroxidase.

2.7 EPITOPE MAPPING OF MONOCLONAL ANTIBODIES:
2.7.1 SDS-PAGE and Western Blotting: SDS-PAGE was performed by the method of Laemmli (1970) using a mighty small II dual slab gel apparatus. BhCG (50µg) was dissolved in treatment buffer (0.125M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol) without or with 10% 2-mercaptoethanol, boiled for 5min and run on a 12.5% SDS-PAGE. The protein was transferred to nitrocellulose (NC) filters according to
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Towbin's method (Towbin et al., 1979). Transfer was performed at 50V for 3 hr using a TE 22 mini transphor apparatus (Hoeffer Scientific, USA). After the transfer a part of NC filter was cut and stained with 0.1% amido black 10-B. Rest of the NC filter was blocked with 3% BSA in 50mM PBS for 2hr at room temperature and washed thrice with PBS containing 0.1% Tween-20. NC filter was cut in to strips and probed with MAbs as described previously (Gupta, et al., 1990). Incubations were done at room temperature for 2hr and filters washed at least thrice in between the steps with PBST. NC filter strips were incubated with MAb ascites (1:1000). Bound mouse antibodies were detected using peroxidase conjugated goat anti-mouse IgG (1:3000). Color was developed using 4-chloronapthol (33mg of 4-chloronapthol dissolved in 10ml of methanol, diluted 4 times with PBS containing 80μl of H2O2). Reaction was stopped by washing the strips with 0.1% sodium azide.

2.7.2 Reduction and Carboxyamidomethylation of BhCG: BhCG was reduced and carboxyamidomethylated according to the method of Kessler et al. (1979a). It was dissolved in 10ml of 0.025N HCl to give a final concentration of 1μM and boiled at 80°C for 1hr. To this was added 20ml of denaturing solution (12M urea, 700mM Tris-HCl, 3% EDTA, pH 8.5) containing 100mg of DTT and incubated under N2 for 30min at 37°C. The exposed SH groups were blocked by adding 228mg of iodoacetamide and the mixture was kept in dark for 60min at room temperature. The pH of the solution was maintained between 8 to 8.5. After the reaction, solution was concentrated to 3ml on a Amicon ultrafiltration apparatus using a filter with molecular weight cutoff of 5000. The concentrate was desalted on a sephadex G-25 column using 0.002N acetic acid, having a
flow rate of 40ml/hr. Fractions of 1ml were collected and absorbance monitored at 280nm. All fractions within the protein peak were checked on SDS-PAGE and those showing βhCG were pooled and lyophilized. Reactivity of MAbs with RCM βhCG was checked in a direct binding ELISA. Microtitration plates were coated with either βhCG or RCM-βhCG (100ng/well) and MAbs were used at suboptimal amount.

2.7.3 Reactivity of MAbs with βhCG Peptides -38-57, 109-118 and 169-145: This was checked in direct binding EIA. All peptides were coated on to microtitration plates at a concentration of 1μg/well. MAbs at saturating amounts were used and bound antibodies were revealed with peroxidase conjugated goat anti-mouse IgG. Antibodies against respective peptides were used as positive controls and were revealed using peroxidase conjugated Protein-A.

2.8 ANALYSIS OF EPITOPE SPECIFIC ANTIBODY RESPONSES IN WOMEN IMMUNIZED WITH HSD-VACCINE:

2.8.1 Vaccine, Immunization Schedule and Sera: The sera analyzed in this study were from women enrolled by informed consent for the Phase II efficacy trials of the HSD vaccine. These trials were approved by the Drug Regulatory Authority and the Institutional Ethics Committees. The women were of proven fertility, having normal ovulatory cycles and active sex life with partners with normal semen parameters. Preparation of HSD and its conjugation to TT or DT has been described earlier (Gaur et al., 1990). Primary immunization consisted of 3 injections of the vaccine given intramuscularly at 6 week intervals. While the first injection consisted of a mixture of 150μg each of HSD-TT and HSD-DT
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conjugates and 1.0mg of SPLPS, the second and third injection consisted of 300μg HSD-DT and HSD-TT respectively. Boosters were given with HSD-DT or HSD-TT at an average of 3 months to keep the antibodies above 50 ng/ml (bioneutralization capacity), the threshold fixed for studying efficacy. All sera samples were kindly provided by Drs G. P. Talwar, Om Singh and Rahul Pal, NII.

2.8.2 Antibody Responses Against Epitopic Domains Defined by MAb 206, MAb 218, MAb 357-2 and MAb P223: These were determined in two types of inhibition EIAs and for convenience named forward inhibition EIA (FIE) and reverse inhibition EIA (RIE). Assay conditions were similar to those described earlier. In both assays, microtitration plates were coated with hCG at a concentration of 100ng/well in PBS and all steps till blocking were similar.

a) Reverse Inhibition EIA (RIE): Saturating amount of MAbs -206, -357-2, -218 and -P223 were added to block their respective epitopic domains. This was followed by the addition of human serum samples (10μl/well) diluted in PBST containing 0.25% BSA (PBST-BSA), at a final dilution of 1:50. Bound human antibodies were detected with peroxidase labeled rabbit anti-human Ig. In control wells instead of MAb, PBST-BSA was added. Wells which were not coated with antigen were used to determine background binding of human sera to the plates. Percent inhibition was calculated as

\[
\text{% Inhibition} = 100 \times \left( 1 - \frac{\text{O.D. in presence of MAb}}{\text{O.D. in absence of MAb}} \right)
\]

b) Forward Inhibition EIA (FIE): Increasing dilutions of human serum
samples (diluted in PBST-BSA) were added in duplicate to microtitration plates coated with hCG. After incubation, without washing the plate, an appropriate dilution of peroxidase labeled MAb(10μl/well), giving an OD of approximately 1.0 in absence of any serum sample was added. Normal human serum and preimmune serum from the respective subject were used as controls. At least 2 samples from an earlier assay were carried over in subsequent assays as positive controls. Percent inhibition was calculated as

\[
\text{% Inhibition} = 100 \times \left(1 - \frac{\text{OD test serum}}{\text{OD control serum}}\right)
\]

The dilution of human serum resulting in 50% inhibition in the binding of peroxidase labeled MAb was determined by regression analysis and was designated as ED50.

c) Reactivity Against βhCG Loop Peptide 38-57 and Carboxy Terminal Peptide 109-145: This was determined in direct binding EIA. Microtitration plates were coated with βhCG loop peptide 38-57 and CTP at a concentration of 500ng/well and 1μg/well respectively (this was optimized by carrying out checkerboard titrations) in PBS and non specific sites blocked with BSA as described above. Human sera were diluted (1:25) in PBST-BSA and were added in duplicates. MAb 357-2 and Rabbit anti-CTP were used as a positive controls for respective assays. The bound human antibodies, MAb 357-2 and rabbit anti-CTP were detected with peroxidase labeled rabbit anti-human Ig, rabbit anti-mouse Ig and Protein-A respectively. Color was developed and
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absorbance taken at 490nm.

d) Anti-hCG Antibody Titres: These were determined in direct binding EIA. The dilution of human sera giving an absorbance of 1.0 was determined and denoted as Antibody Units (AU).

2.9 IgG Subclass Distribution of Anti-hCG and Anti-DT Antibodies in Women Immunized with HSD Vaccine: A direct binding EIA was used for this purpose. All assay conditions were similar to those described earlier. Microtitration plates were coated with 100ng/well of hCG or DT. After blocking with BSA, increasing dilutions of human serum samples (diluted in PBST-BSA) were added in duplicates. Mouse MAbs to human IgG1 (HP6012), IgG2 (HP 6014) IgG3 (HP6050) and IgG4 (HP6101) were used at dilutions of 1:1000, 1:1000, 1:1000 and 1:2000 respectively. Bound mouse antibodies were detected using human adsorbed goat anti-mouse IgG-Fc conjugated to peroxidase at 1:10,000 dilution. In all plates, respective human IgG subclass myeloma proteins captured on goat anti-human IgG-F(ab)_2 were used as positive controls. At least 2 serum samples from previous assays were carried over in next assay to determine interassay coefficient of variation. Dilution of human sera giving an O.D. of 0.5 was determined and designated as antibody units.

2.10 ANALYSIS OF ANTI-hCG ANTIBODY REPERTOIRE IN ANIMALS:

To study the antibody repertoire against hCG in animals, rat was used as a model system.

2.10.1 Immunization Schedule: Adult female Wistar rats (n=8) bred in the Institute's small animal facility and fed ad libitum were immunized
with the HSD vaccine formulation adsorbed on alum. Primary immunization consisted of 3 injections of the vaccine given intramuscularly at 6 week intervals (days- 0, 42 and 84). The first injection consisted of a mixture of 5 μg each of HSD-TT and HSD-DT conjugates and 250 μg of SPLPS, the second and third injection consisted of 10μg HSD-DT and 10μg HSD-TT respectively. A booster was given on day 125 with 10μg of HSD-DT. Animals were bled retro-orbitally on days 31, 50, 93, 107, 123 and 133. Serum was separated and stored at -70°C till use.

2.10.2 Analysis of Anti-hCG Antibody Repertoire: Pooled bleeds from 8 animals obtained at various time points and individual bleeds on day 93 were analyzed for anti-hCG antibody titres, reactivity against βhCG loop peptide 38-57, CTP, and MAb 206 defined epitopic domain. Methods used were similar to those described earlier for human subjects with few modifications. Reactivity against βhCG loop peptide was determined at a serum dilution of 1:50. The rat sera were titrated against CTP and reactivity was expressed in Antibody Units. The bound rat antibodies were detected with peroxidase conjugated goat anti-rat IgG.