Contraception by evoking a specific immune response against antigens crucial for fertility is a feasible proposition. HCG, a pregnancy specific hormone, is synthesized at the preimplantation stage (Fishel et al., 1984) and is essential for the sustenance and maintenance of early pregnancy (Csapo et al., 1972). Immunocontraceptive vaccines based on hCG have reached an advanced stage of development with the initiation of human clinical trials (Talwar et al., 1976; Jones et al., 1988; Talwar et al., 1990). One such vaccine based on the HSD of αLH associated with the βhCG and conjugated to either TT or DT (αLH-βhCG-TT/DT or HSD vaccine) (Talwar et al., 1988) has demonstrated its efficacy in preventing pregnancy in the immunized human volunteers (Talwar et al., 1992; 1993). Anti-hCG antibodies can prevent pregnancy by neutralizing hCG bioactivity or by a direct action on the embryo (Hearn et al., 1988). However, all antibodies against hormones, though immunoreactive, do not necessarily abrogate the bioactivity of the hormones (Beall et al., 1973; Louvet et al., 1974; Matsuura et al., 1979).

We were interested to delineate epitope specific antibody responses against hCG in women immunized with the HSD vaccine. For this purpose two approaches were followed; first being the use of MAbs as probes and second, the use of synthetic peptides. Inhibition assays employing MAbs and polyclonal antibodies to native protein have been used to map antibody responses to meningococcal outer membrane epitopes (Mandrell and Zollinger, 1989), major epitope p24 of human Immunodeficiency virus type 1 (Janvier et al., 1991), human growth hormone (Etcheverrigaray et al., 1988) etc.

Thus a panel of anti-hCG monoclonal antibodies was generated and
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characterized. Based on the reactivity patterns against hCG, αhCG and βhCG in competitive RIA and in sandwich ELISA, the panel of antibodies broadly recognized 5 different epitopic domains on hCG (Fig. 6). MAbs: -206, -222, -234, -62, -71 and -P3W80 recognized overlapping/similar epitopes within an epitopic domain common to βhCG and hCG. MAb-357-2 recognized epitopic domain defined by the βhCG loop peptide 38-57. This epitope was distinct from that recognized by the group of 6 antibodies. None of these antibodies recognized the βhCG 38-57 loop peptide in a direct binding EIA (data not shown). Also this peptide could not inhibit the binding of MAb 206 to hCG (Fig. 5). MAb 218 recognized a unique conformational epitope on hCG contributed by the α- and β-subunits. A previously characterized MAb-P223 (Gupta et al., 1985b) represents a epitopic domain on αhCG. An epitope masked by the association α and β-subunits was recognized by MAb 67. This antibody reacted only with free βhCG. This antibody was not studied further as its epitope was not relevant for this study.

Four MAbs (-206, -357-2, -218 and -P223), representative of 4 epitopic domains were used as probes. The reactivity of these antibodies was checked with the 2 HSD-vaccine formulations, HSD-TT and HSD-DT, to see whether these epitopes were present on the vaccine. Interestingly only MAb 206 and MAb 357-2 could recognize the vaccine whereas MAb 218 and MAb P223 failed to do so (Fig 7). Using these 4 MAbs, 2 types of inhibition ELISAs were developed, RIE and FIE. In these assays preincubation was either with MAbs (RIE) or with the immune sera (FIE). In the RIE, no significant inhibitions were obtained with MAbs -357-2, -218 and -P223 (Fig. 8). Lack of antibody response against epitopes
defined by MAb 218 and MAb P223 is because of the absence of these epitopes on the vaccine. Inhibitions below 20% (2 samples above 20%) by MAb 357-2 seem to be insignificant as none of the serum samples tested recognized β-hCG loop peptide in the direct binding EIA even at a serum dilution of 1:10, indicating an absence of antibody response against this region (Table 4). However, MAb 206 could inhibit significantly the binding of polyclonal sera to hCG. Dominance of antibodies against MAb 206 defined region and the absence of antibodies against β-hCG loop peptide 38-57 is not a random phenomenon as is seen from the analysis of sequential bleeds in RIE as well as FIE and direct binding EIA. Poor immunogenicity of one of the receptor binding domains of βhCG imparts the hormone natural protection from the immune system. Whether the poor immunogenicity of this domain is because of epitope specific tolerance or clonal dominance of B cells recognizing other epitopic domains, such as those defined by MAb 206 needs to be addressed. Interestingly, analysis of secondary B cell repertoire for rat cytochrome c in BALB/c mice has shown that the major antigenic region of cytochrome c is located on the surface opposite to that containing the exposed heme crevice (Jemmerson and Blankenfeld 1988, Jemmerson and Johnson 1991). The heme crevice is the active site on cytochrome c, which interacts with its partners within the respiratory chain (Ahmed et al., 1978, Ferguson-Miller et al., 1978).

Antibody response against an epitopic domain on hCG represented by the carboxy terminal end of βhCG was probed using the synthetic peptide β109-145. The CTP is unique to hCG and is absent in hLH (Pierce and Parsons, 1981). The CTP conjugated to TT/DT also forms the basis of
Another anti-hCG vaccine that is in clinical trials (Jones et al., 1988). The epitopic domain defined by CTP was poorly immunogenic in humans as, of the 28 subjects screened only 16 had antibodies against this domain detectable at a serum dilution of 1:25 (Table 5). Also this response was highly variable and had no correlation with the total anti-hCG antibody titres. The analysis of sequential bleeds from five women showed that antibody response in three subjects during the initial period of immunization was better than the later half (Fig. 12), whereas in one subject (618) there was no response throughout the immunization schedule. This might be because of the immunodominance of epitopic domains, such as those defined by MAb 206 or because of affinity maturation of antibodies against CTP, which results in the failure to recognize the linear peptide on ELISA plates. The former might be the case for several reasons. Bidart et al. (1987a) have shown that most of the epitopes on CTP are linear epitopes with the epitope represented by the sequence 110-116 being most immunodominant and with the increase in affinity, the linear epitopes should be recognized better. Poor immunogenecity of CTP on HSD vaccine is a highly significant finding towards understanding the antibody repertoire responsible for the efficacy of HSD vaccine. Phase-I clinical trials of vaccine based on synthetic CTP in humans has revealed the development of antibodies against this determinant (Jones et al., 1988). However the data on efficacy of this vaccine in humans is still awaited. Our results demonstrate for the first time that contraception by HSD vaccine can be achieved despite lack of antibodies against the CTP in women. However, the relative merit of anti-CTP antibody titers to achieve contraception in responder versus
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nonresponder women needs further evaluation.

Ehrlich et al. (1985) had used a competition RIA to study the effect of an autoimmune human antiserum to hCG (Thau et al., 1983) on the binding of 9 MAbs to hCG. In this case it was seen that antibodies recognizing epitope region III (conformational epitope formed at the interface of α and β subunits) dominated the response. Whereas our studies indicate immunodominance of epitope recognized by MAb 206, contributed by the β subunit of hCG. This may be because the antigens with which the immune response against hCG is generated in two situations are different. Also an autoimmune response to hCG is a rare phenomenon and is mostly observed in patients to whom hCG is administered for therapeutic purposes (Claustart et al., 1983; Braunstein et al., 1983).

To see whether the antibody repertoire generated against hCG in women, where it is essentially a self molecule is different in situations where it is foreign, rats were immunized with the HSD-vaccine. Rats were used as a model system as it has been conclusively demonstrated that rats do not have CG like material (Wurzel et al., 1984). Also rat LH β gene has been cloned and sequenced and the amino acid sequence of rat LH is known (Jameson et al., 1984). Although their is a high sequence homology between rat βLH and βhCG (Ryan et al., 1987), rat LH also lacks the C-terminal extension of hCG. The immunization schedule followed for humans was used and antibody responses against 3 epitopic domains defined by MAb 206, βhCG 38-57 loop peptide and the CTP were probed.

As in women, antibody response against epitopic domain on hCG defined by MAb 206 was also detected in rats. Moreover, the titre of antibodies
against this domain correlated well with the total anti-hCG antibody titres. This suggests promiscuous nature of this domain on hCG. This domain is also immunogenic in mice, as most of the MAbs recognize overlapping epitopes within this domain. However, immunogenicity of βhCG loop peptide 38-57, which is one of the receptor binding area of βhCG was poor in rats as well. Only 2 animals out of 8 had antibodies against this domain.

However, CTP was highly immunogenic in rats. Of the 8 animals tested, all had generated a good immune response against CTP. A good antibody response against CTP in rats in contrast to humans might be because CTP is recognized as a foreign antigen, being given that this sequence is not present in rat βLH and that rats have no CG or its counterparts (Wurzel et al., 1984; Jameson et al., 1984).

Absence of antibodies against the loop peptide and poor immunogenicity of CTP in women suggest that the bioneutralization of hCG by human polyclonal antibodies is achieved by blocking other receptor binding domains of βhCG or by steric hindrance. One such region may be the ones defined by MAb 206. A good correlation between the antibody titer against the epitope defined by MAb 206 and bioneutralization capacity has been observed (Fig. 10).

Interestingly the epitopic domain defined by MAb 206 is a non receptor binding domain on hCG as 125I-MAb 206 could recognize hCG complexed to its receptor (Table 8). Antibodies recognizing non receptor binding domains bring about the inhibition of hCG-receptor interaction either by steric hindrance or by inducing a change in the conformation of hCG, so that the affinity of hCG for its receptor is reduced (Armstrong et
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al., 1986). To check the bioneutralization capacity of MAbs recognizing similar or overlapping epitopes within the MAb 206 defined epitopic domain radioligand receptor assays were done. Based on dose response curves the ability of MAbs at a concentration of 1µg/ml to inhibit hCG-receptor interaction was determined. Interestingly high affinity antibodies were more potent in inhibiting binding of hCG to its receptor. A good correlation was observed ($r = 0.97$, $p<0.01$) between the affinity of anti-hCG MAbs and the inhibition of binding of hCG to its receptor (Fig. 17). However, in vivo studies, could not confirm this phenomenon. Although all MAbs could significantly inhibit ($p<0.001$) the hCG induced uterine weight gain, significant differences were not observed between the ability of different MAbs to inhibit the weight gain. One of the reasons for this might be, that MAbs were in excess amount and need to be tested at suboptimal concentrations. However, this information is highly significant as an hCG specific response within MAb 206 defined epitopic domain would be the key for the success of HSD contraceptive vaccine. Attempts were made to map the epitopic domain defined by MAb 206. However, MAb 206 did not recognize RCM-βhCG (Fig. 15) indicating that this epitope was a discontinuous epitope contributed by different regions of βhCG. Although the precise immunochemical location of this domain is not known, based on the available literature few predictions can be made. The epitopic region described in this study lies probably in the first 110 amino acids of βhCG as none of the MAbs recognized the βhCG peptides 109-145 and 109-118 in direct binding ELISA (data not shown). Also MAb 206 does not recognize the βhCG loop peptide 38-57, which is implied as an important receptor binding domain of βhCG. This
finding is reconfirmed as this MAb can recognize hCG complexed to its receptor. Thus the receptor binding domains of BhCG can be excluded from the epitopic domain of MAb 206. Bidart et al (1987b) have postulated the involvement of BhCG peptide 82-105 in the epitope of an anti-hCG MAb (FBT 10) which can recognize hCG complexed to its receptor and this peptide stretch might be involved in the epitopic domain recognized by MAb 206. However other regions on BhCG cannot be ruled out as BhCG peptide 82-105 had only weakly inhibited MAb FBT 10 from binding to hCG. Also a disulfide bridge between amino acids 9 and 83 (Ryan et al., 1987), probably brings the N-terminal regions of BhCG in proximity with regions around amino acid 83. These observations indicate that amino acids 1-40 and 60-105 probably define the epitopic region on hCG recognized by MAb 206 and which is exposed on hCG coupled to its receptor.

Human IgG can be divided into 4 major subclasses, IgG1, IgG2, IgG3 and IgG4. They constitute approximately 65, 30, 5 and 4% of the total IgG respectively. Each subclass has different biological and physicochemical properties. Various IgG subclass restrictions have been reported for immunoglobulin response to autoantigens. Predominant IgG1 and IgG4 responses have been shown against myeloperoxidase (Esnault et al., 1991) and against thyroglobulin in patients with autoimmune thyroid disease (Devey et al., 1989). Whereas preponderance of antibodies of IgG1 and IgG3 sub classes to cardiolipin have been demonstrated in patients with systemic lupus erythematosus (Loizou et al., 1992). The IgG2 subclass response has been shown to be restricted to carbohydrate determinants (Da Costa et al., 1993). It was of interest to
study, whether such isotype restrictions were also present in antibody response against hCG in human subjects immunized with a β- hCG based contraceptive vaccine, as hCG is essentially a self molecule. Such studies are also important from the point of view of understanding the role of various subclasses in the bioefficacy of the vaccine. Also distribution of IgG subclasses provides an insight into the immunological processes involved. Anti-hCG antibody response was predominantly of IgG1 subclass (Fig. 19) with a mean titer of 537.94±560 Antibody Units (AU). The other 3 subclasses showed considerably lower mean levels (IgG2=16.46±8.33, IgG3= 3.22±8.48, IgG4=56.65±82.60 AU). A good correlation was observed between the anti-hCG IgG1 antibody titers (r=0.57, p<0.01) and the bioneutralization capacity of sera. However, bioneutralization capacity of the sera from subjects capable of inducing IgG4 response was not significantly different from subjects not showing IgG4 antibodies (Fig. 22), thereby suggesting that IgG4 antibodies do not contribute significantly in neutralization of hCG bioactivity. Our results suggest anti-hCG IgG1 antibodies to be critical for the vaccine efficacy. However, the effect of purified anti-hCG IgG1 and IgG4 antibodies should be determined in vivo, as apart from inhibition of hCG-receptor interaction other mechanisms have been implied in the bioefficacy of anti-hCG antibodies (Hearn et al 1988). These studies would be significant as IgG4 antibodies do not fix complement and have been shown to have inhibitory effect on sperm immobilizing IgG1 antibodies (Tsuji et al., 1993).

Similarly a dominant IgG1 response was observed against diphtheria toxoid (DT) which has been used as one of the carrier protein in the
vaccine (Fig. 20). To compare the relative proportion of IgG4 antibodies with respect to IgG1 antibodies against both antigens IgG4/IgG1 ratios were calculated (Fig. 21). These ratios suggest that in 66.66% of subjects the IgG4 responses against both self and foreign antigen administered cognitively on the vaccine were similar. However, in 13% of the subjects there was a predominant anti-DT IgG4 as compared to anti-hCG IgG4 response. Whereas in 20% of the subjects an anti-hCG IgG4 response was predominant as compared to anti-DT IgG4 response.

The role of various cytokines in isotype switching is well documented. IL-4 mediates an isotype switch to IgG4 and IgE (Ishizaka et al., 1990) whereas IL-2 plays an important role in the IgG1 response (Armitage et al., 1993). It has been also shown that IL-2 (Nusslein and Spiegelberg, 1990) and IFN-\(\gamma\) (Spiegelberg et al., 1991) inhibit IL-4 induced IgG4 secretion. While IL-2 and IFN-\(\gamma\) are products of Th1 cells, IL-4 is made by Th2 cells (Mosmann and Coffinan, 1989). Although human CD4\(^+\) T cells cannot be clearly distinguished in to Th1 and Th2 subsets (Maggi et al., 1988), a recent report has provided evidence for excessive Th2 CD4\(^+\) subset activity in vivo, resulting in to a predominant IgG4 and IgE response (Field et al., 1993).

It would be interesting to investigate whether subjects having a predominant IgG1 response against hCG and DT are activating a subset of T cells making predominantly IL-2, while a mixed IL-2 and IL-4 response is seen in subjects with detectable IgG4 against both antigens. Also of interest would be to study the cytokine profiles of helper T cells from subjects giving a dichotomy in IgG4 response against hCG and DT.