DISCUSSION
In response to its mandate to broaden choices in family planning, the World Health Organization (WHO) has been engaged in improvement of existing and in the development and assessment of new methods of fertility regulation for more than 20 years. Through the research carried out by its Task Force on Vaccines for Fertility Regulation, the WHO has established the feasibility of developing a fertility-regulating vaccine directed against hCG. The ongoing research is directed at improving the safety, efficacy, cost-effective availability and the convenience of use of the anti-hCG vaccine approach.

In our efforts to make use of viral vectors for expression and delivery of the hCG vaccine, in the present study an E1-substituted recombinant human adenovirus was made which was capable of synthesizing and targeting the beta-subunit of hCG to the cell surface. The recombinant adenovirus induced hCG bioneutralizing antibodies in experimental animals. Over the last 5 years adenovirus vectors have gained considerable popularity for the development of recombinant vaccines and a number of antigens have been expressed using these vectors. The popularity of adenovirus as a recombinant viral vector is largely due to the successful and safe immunization of millions of US military recruits with enteric-coated Ad4 and Ad7 as a prophylactic measure against acute respiratory disease outbreaks. Besides, E1- substituted adenoviruses are replication-defective in vivo and thus do not pose the risk of being transmitted from the vaccinees to unintended subjects. This feature makes adenovirus an attractive vector for the development of a recombinant virus based birth control vaccine.

Adenovirus vectors are being used increasingly for a variety of applications in vaccination and gene therapy. In both cases the choice of the vector and the promoter are known to determine the outcome of the experimental approach. For vaccination, the majority of work has been done using replication-competent Ad vectors which carry foreign gene cassettes in the E3 region of the genome since it happens to be non-essential for viral replication (Graham and Prevec, 1992). Although not needed for viral replication, Ad E3 region codes for a suite of proteins which interact with the immune system in various ways (Wold and Gooding, 1990). Thus, deletion of the E3 19 K glycoprotein gene in Ad5, induced
severe immune pathology in cotton rats (Ginsberg et al., 1989), raising concern about the safety of such vectors. In fact, if the data obtained in cotton rats were to extrapolate to infection in humans, replication-competent, E3-substituted vectors may not be suitable for use. An alternative approach is to develop recombinants in which foreign gene expression cassettes are inserted in the E1 region of the Ad genome. As E1 codes for proteins which transactivate other viral and cellular promoters (Liu and Green, 1994), viruses which lack this region are replication-deficient in all but human 293 cells which provide the missing functions in trans (Graham et al., 1977). This strategy therefore offers advantages from a safety viewpoint.

In E3-substituted viruses it has not proved necessary to introduce a promoter to drive foreign gene expression as genes inserted in the left-to-right orientation are efficiently expressed, either from the upstream major late promoter (MLP) or form the E3 promoter in the viral genome (Both et al., 1993; Morin et al., 1987). However, most E1 replacement vectors designed for high-level expression, have traditionally made use of the MLP and sequences from the tripartite leader at the 5'-end of the gene to be expressed, though at least one vector has been constructed that used the E1A promoter (Ballay et al., 1985). At present, human cytomegalovirus (hCMV) promoter is considered to be the promoter of choice when designing E1-substituted Ad vectors (Jacobs et al., 1992; Morsy et al., 1993; Xu et al. 1995). Despite being replication-deficient, E1-substituted Ad recombinants express enough protein that may be necessary for inducing a desired immune response. For example, an E1-substituted adenovirus expressing flavivirus non-structural protein induced a significant protective immune response in vaccinated mice (Jacobs et al., 1992). Thus, the DNA coding for the βhCG-VSV fusion protein was placed under the transcriptional control of the hCMV immediate early promoter for making an E1-substituted, replication-defective recombinant human adenovirus. Orientation of the expression cassette with respect to the E1 transcription unit is probably not important for expression (Berkner and Sharp 1984; Davidson and Hassel 1987) although Xu et al. (1995) found that the expression of the rotavirus VP7sc gene in an E1-substituted recombinant adenovirus using hCMV promoter from the rightward facing cassette was
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consistently better than that from the one facing leftward. In present study the $\beta hCG_{cs}$ expression cassette was inserted in the E1 transcription unit in the rightward orientation.

The interest in generating $\beta hCG$ fusion proteins stems from the fact that conjugation of this subunit with an immunogenic carrier protein like tetanus toxoid (TT) renders this self protein antigenic in humans (Talwar et al., 1976). For preparation of the hCG vaccine, the $\beta$ subunit of hCG is conjugated to TT using the periodate and maleimide ester methods (Gaur 1985). The $\beta hCG$-TT conjugates prepared by different procedures, have been shown to give variable immune response. This clearly suggests the influence of the conformational constraints imposed by the conjugation chemistry, on the exposition of antigenic epitopes. It is highly probable that not all $\beta hCG$ molecules attach to TT molecules in an identical fashion and different conjugates of $\beta hCG$-TT may exhibit subtle structural heterogeneity. Thus, in application where the conjugate is to be eventually used as a contraceptive vaccine, it is of paramount importance to have a homogenous population of the $\beta hCG$ conjugate molecules, where all the molecules have a uniform conformation and are assured to expose the correct antigenic epitopes.

Among the several possible ways to conjugate $\beta hCG$ with a carrier protein, an attractive approach would be the genetic approach, in which the DNA encoding $\beta hCG$ is linked directly to that coding for a carrier protein. Such an approach of engineering gene fusions to bring about the attachment of amino terminal portion of one protein to the carboxy terminal portion of another, while retaining the functional activity of one or both proteins, has been exploited in the past to address a number of biological problems (Guan et al., 1985; Fukuda et al., 1988; Guan et al., 1988). Thus, genetically linking the $\beta hCG$ DNA with that of a carrier, such that the two are translatable into a chimeric protein, would preclude many of the disadvantages inherent in the conventional conjugation methods. Mutagenesis studies have shown that the N-terminus of $\beta hCG$ is involved in receptor binding whereas its C-terminus is not important for subunit association or receptor binding (Chen et al., 1992; Keutmann, 1992). Thus, chimeric $\beta hCG$ containing a carrier moiety at its C-terminus, produced by recombinant
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DNA means, has the advantage of consistency and a recombinant virus producing this fusion protein may have the added advantage of being economical and relatively easy to deliver.

Membrane localization of protein allows the immune system a better access to the antigen (Andrew et al., 1991). That there is an increase in antibody titer in response to cell-surface-anchored antigen is based on the idea that a threshold number of T or B cell antigen receptors need to bind to the antigen in order to activate the cells. Antigens anchored on the surface of an infected cell might allow better multipoint binding of antigen to the receptor on the B cell. This would occur, especially if the antigen accumulates in patches on the cell surface. For helper T cells, patches of infected cell membrane containing multiple copies of the antigen could be taken up by antigen presenting cells, so that a more effective concentration of antigen/MHC complex is displayed on the surface of the antigen presenting cell. The possibility of the cell surface-anchored proteins being protected from degradation to a greater extent than either intra-cellular or secreted proteins can also add to the improved immunogenicity of cell membrane-anchored antigen. There are numerous examples in literature where cell surface expression of an antigen has been shown to increase its immunogenicity by several orders of magnitude. For example, Langford et al. (1986) demonstrated that the immunogenicity of the Plasmodium falciparum S antigen was enhanced significantly when the cDNA clone was engineered to contain the murine membrane immunoglobulin transmembrane sequence which yielded a protein localized to the cell surface. Enhanced immunogenicity was also obtained for the rotavirus glycoprotein VP7 which was directed to the plasma membrane by addition of the influenza virus hemagglutinin leader and anchor sequences (Andrew et al., 1990). Similarly, membrane anchoring of the rotavirus inner capsid protein VP6 with a mouse gamma globulin anchor domain was shown to enhance its immunogenicity (Reddy et al., 1992). Srinivasan et al. (1995) have also shown that fusion of the vesicular stomatitis virus (VSV) glycoprotein transmembrane and cytoplasmic domains to the C-terminus of βhCG leads to its cell surface localization and enhanced immunogenicity. In present study too, cell surface expression of the βhCG by recombinant adenovirus was achieved by fusing the VSV glycoprotein transmembrane and cytoplasmic domains to the C-
terminus of βhCG. The presence of the βhCG fusion protein was demonstrated on the cell surface of 293 cells that are replication-permissive for E1-deficient adenovirus as well as in HeLa, COS-7 and MRC-9 cells where E1-deficient adenovirus can not replicate. Detection of βhCG fusion protein on the cell surface by monoclonal antibody P3W80 indicates that the specific epitope recognized by this monoclonal is preserved despite its fusion to a sequence from the VSV glycoprotein and its subsequent cell surface localization.

The levels of βhCG fusion protein (βhCGCS) synthesis in 293 cells by the recombinant adenovirus were studied by RIA. The expression could be detected as early as 6 h p.i. and a peak expression level of ~300 ng βhCG fusion protein/10⁶ cells was observed at 24 h p.i. after which a gradual decline in the levels of expression was noted. Recombinant adenoviruses have been shown to express foreign proteins with varying efficiency. For example, envelope glycoprotein of HIV type I has been expressed using an E3-substituted recombinant adenovirus vector to a level as high as 2-5 mg/litre (Chanda et al., 1990), while in case of transmissible gastroenteritis virus spike protein, expression levels ranged from 100 ng to 10 μg of truncated or native forms of the spike protein from 10⁶ cells infected infected with an E3-substituted recombinant (Torres et al., 1995). Similarly, depending upon the cell type used an E1-substituted recombinant adenovirus expressed varying amounts of hepatitis B surface antigen (HBsAg) ranging from 0.4 μg to 11 μg per 10⁶ cells (Levrero et al., 1991). The β subunit of hCG has been expressed in a variety of eukaryotic systems, however, the expression levels have always been poor (Reddy et al., 1985; Chakrabarti et al., 1989). It is, therefore, not entirely surprising that Ad system synthesized lower amounts of βhCGCS. In the present study the βhCG was expressed as a membrane-anchored chimeric protein. For estimating the amounts of the fusion protein, the virus-infected cells were lysed by three cycles of rapid freeze-thaw, passed through a 26 gauge needle and the supernatant collected after centrifugation was used in RIA. It is possible that some of the membrane-anchored protein is lost in the cell pellet. This was, though, not investigated. Besides, our results may not reflect the accurate measurement of the amount of βhCG fusion protein as these were measured using an RIA designed for the estimation of βhCG. Thus amounts of the fusion protein recorded in the assay
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may only be read as βhCG equivalent and not as absolute amounts, which may be relatively higher.

In addition to high copy number of the gene, the level of foreign protein production in mammalian cells can be boosted by increasing the number and stability of the RNA transcripts and by augmenting their efficiency of translation. The mechanism by which eukaryotic ribosomes recognize and bind to a unique initiation site on an mRNA is not yet completely understood. A scanning model for the initiation of protein synthesis has been postulated by Kozak (1978, 1980) which proposes that the 40S subunit of ribosome initially binds at or near the capped 5'-end of the mRNA and subsequently migrates along the mRNA until it encounters an AUG codon. According to this model, the initiator codon is simply the AUG triplet which is closest to the 5'-end of mRNA. To explain exceptions to such an initiation, the model has been expanded to include effects of the 5' proximal AUG codon's on the translation efficiency of the neighbouring sequence. Thus, an ideal sequence context in higher eukaryotes has been suggested to be GCC(A/G)CCAUGG, with the presence of a purine residue in the −3 position relative to the AUG codon having the greatest influence on efficiency of initiation of translation, and a purine in the +4 position being next most important. According to the Kozak model, then, if the sequences flanking the first AUG codon present a so-called favorable context, the 40S subunit will stop and associate with a 60S subunit of the ribosome, and protein synthesis will initiate uniquely at that site. Thus, Lamarche et al. (1990) demonstrated that a Kozak-modified construct (G at position −3 of translation initiation site) made substantially increased amounts of herpes simplex virus (HSV) ribonucleotide reductase subunit 2 using an adenovirus vector. Similarly, Johnston and Rochon (1996) have recently shown that use of a favorable initiation codon context improved the expression of a 21 K protein of cucumber necrosis virus.

According to the Kozak model, the 5' proximal AUG codon of the βhCG mRNA (CCAAGGAUGG) occurs in an initiation context substantially different from the Kozak consensus sequence for ribosome binding. An attempt was, therefore, made to generate a
favorable initiation context for βhCG (GCCGCAUGG) by site directed mutagenesis using an approach based on PCR. Our studies revealed that the levels of βhCG synthesis in HeLa and Véro cells transfected with the Kozak modified construct (pCEPKZβhCG) were similar to those seen in cells transfected with the native construct (pCEPβhCG). Thus the presence or absence of the initiator AUG codon in a consensus favorable sequence context had no discernible effect on the amounts of βhCG synthesis. Recently, Escaich et al., (1995) have reported that cells expressing the HIV-1 RevM10 gene with an optimized Kozak-consensus sequence produced comparatively less Rev protein than the cells carrying the native RevM10 gene. However, they suggest that the reduction in Rev protein was caused by slightly lower steady-state Rev RNA levels and not as a result of reduced translation of the mRNA in cells transduced with the Kozak-modified construct.

Remarkable differences were observed in the levels of βhCG synthesis in different cell lines transfected with pCEPβhCG. Similar results have been reported for chloramphenicol acetyl transferase (CAT) protein expression levels in different cell types employing hCMV promoter (Sheay et al., 1993). The higher levels of βhCG expression were observed in cell lines of human (HeLa and 293) or primate origin (COS-7, CV1 and Véro) than those of the rodent origin (NIH 3T3 and CHO). The hCMV promoter has been shown to be most active in 293 cells (Lin et al., 1994) and least active in NIH3T3 (Sheay et al., 1993) and CHO cells (Lin et al., 1994). Since the promoter strength is determined by the transcription factors that bind to defined elements within a promoter and enhancer and because different cell types are equipped with different sets of transcription factors, most cellular promoters and enhancers are controlled in a tissue specific manner. Thus, heterogeneity in the expression levels may be a reflection of the differential activity of the hCMV promoter in different cell lines. We have, however, not ruled out the possibility of differences in the transfection efficiencies and susceptibility of different cell types to the toxic effects of Lipofectin.

Although levels of βhCG fusion protein synthesis by recombinant adenovirus in vitro were not high, these may be sufficient for induction of the immune response in vivo. To this end, the potential of RAdβhCGcs for inducing anti-hCG antibodies was investigated in Wistar
rats. Immunogenicity studies were conducted in outbred rats so as to minimize the possibility of strain related hyporesponsiveness. Rats were inoculated through intra-muscular, intra-peritoneal or oral routes with three doses of $10^6$, $10^7$ or $10^8$ PFU/animal. In animals inoculated through intra-muscular or intra-peritoneal routes, anti-hCG antibodies could be detected as early as 20 days post-inoculation and the titers gradually increased till the 50th day post-inoculation. However, animals that received the virus through oral route failed to generate an appreciable amount of anti-hCG antibodies. In most instances recombinant adenovirus given orally has been found to induce desired immune response (Lubeck et al., 1989; Prevec et al., 1991; Charlton et al., 1992; Both et al., 1993; Chengalvala et al., 1994). However, there are a few instances where oral immunization with recombinant adenovirus did not elicit an antibody response. For example, Tacket et al. (1992) reported that an adenovirus recombinant synthesizing HBsAg failed to induce anti-HBsAg antibodies when the virus was delivered orally to human volunteers. Similarly, a replication-defective human recombinant adenovirus synthesizing rabies virus glycoprotein induced high titers of virus-neutralizing antibodies, when administered by intra-nasal or sub-cutaneous routes, but the recombinant adenovirus failed to induce neutralizing antibodies when given orally (Xiang et al., 1996). We did not use enteric-coated capsules for the recombinant adenovirus delivery and the virus suspension was administered directly into the gastro-intestinal tract of the rats using a syringe carrying long blunt needle. Whether the mode of recombinant adenovirus delivery into the gastro-intestinal tract can influence its immunogenicity, is not clear.

In rats immunized with RAdβhCGcs, the anti-hCG antibody response was not uniform as animals showed extensive variation in antibody titers including some which did not respond at all. The antibody titers in animals which responded showed a dose dependence; rats inoculated with $10^6$ PFU of the virus made very little anti-hCG antibodies while titers were higher when $10^8$ PFU of the virus per animal was used. Similarly, number of responders were higher with the higher dose in rats inoculated through intra-muscular or intra-peritoneal routes. The capacity of these antibodies to bioneutralize hCG was studied in an in vitro assay by analyzing their ability to prevent binding of hCG to LH/CG receptor. These studies showed
that antibodies from both intra-muscular as well as intra-peritoneal immunizations were capable of neutralizing the hCG bioactivity.

In comparison to immunization studies performed using recombinant vaccinia virus expressing anchored βhCG (Srinivasan, 1991), the immune response generated by the recombinant adenovirus was of a lower magnitude. A single intra-dermal immunization with vaccinia recombinant induced anti-hCG antibodies in a dose dependent manner. At a dose of $10^8$ PFU of the vaccinia recombinant per rat, 100% of the animals developed anti-hCG antibodies. However, at this dose of RAdβhCG cs only 66% of the animals inoculated by intra-muscular route and 83% of the animals inoculated by the intra-peritoneal route made anti-hCG antibodies. As opposed to a replication-competent recombinant vaccinia virus used by Srinivasan (1991), the recombinant adenovirus used in present studies is a replication-defective virus which might require higher dose to elicit an immune response similar to vaccinia recombinant. There are a few reports in literature comparing the immunogenicity of replication-competent versus replication-defective virus vectors for a given system. For example, Taylor et al., (1991) found that the level of neutralizing antibodies and the level of protection against canine distemper virus (CDV) challenge, in rodents, swine, cattle and horses immunized with measles virus antigen expressing vaccinia and canarypox viruses was same. On the other hand the same investigators found that in rodents, swine, cattle and horses, 50% protective dose (PD_{50}) of the replication defective recombinant fowlpox virus expressing rabies glycoprotein was significantly higher than the protective dose obtained with a replication-competent vaccinia virus recombinant expressing the same rabies virus glycoprotein (Taylor et al., 1988; 1991).

In our immunization experiments no mortality or obvious clinical complications were observed in any of the groups of immunized rats until the completion of the study, thereby pointing to the safety of the recombinant adenovirus. A slight drawback, however, may be that a higher dose of recombinant adenovirus may be needed in order to generate an adequate anti-hCG response. However, safety advantage of replication-defective adenovirus reinforces its
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potential for regulating fertility in women. Further characterization of the βhCG-VSV fusion protein expressed by RAdβhCG<sub>CS</sub> must be carried out in terms of its molecular mass, glycosylation and folding pattern, etc., before immunogenicity and efficacy experiments in primates are considered.