Immunotherapy is a form of *biological therapy* or *biotherapy*. It is a treatment that uses certain parts of the immune system to fight diseases, including cancer. This can be done in a couple of ways:

- By stimulating the immune system to work harder or smarter
- By administrering immune system components, such as antibodies or cells

True cancer vaccines are different from the vaccines that work against viruses or bacteria; instead of preventing disease, they are meant to get the immune system to attack a disease that already exists. A cancer vaccine can incorporate cancer cells, parts of cells, or pure antigens. The vaccine increases the immune response against cancer cells that are already resident in the body. It may be combined with other substances (referred to as *adjuvants*) that further help boost the immune response.

Cancer may develop as a result of a reduction in the efficiency of the immune system. Radiotherapy and chemotherapy can kill cancer cells but efficacy rates can vary; bystander killing of healthy cells can result in toxicity, and tumors can also become insensitive to these treatments, resulting in recurrence and metastasis. Immunotherapy holds promise for the treatment for a variety of cancers; where conventional therapy frequently fails to treat metastatic disease, approaches that enhance anti-tumor immunity have the potential to facilitate cure. Anti-tumor effects mediated via activation of host immune responses can be specific and can demonstrate low toxicity (if appropriate targets can be identified), and may also potentially decrease drug resistance, currently a leading cause of therapeutic failure (Whiteside, 2010). However, the search for true target ‘tumor-specific’ antigens has been long and arduous. ‘Tumor-associated’ antigens, which may exhibit relative over-expression in tumor tissues, are also being actively considered (Lewis et al., 2003). A number of potential strategies have been developed to overcome the poor immunogenicity of tumor “self” antigens, including carrier conjugation, co-injection of cytokines and other potent adjuvants, and the tandem repetition of relevant epitopes.

**hCG targeting strategies**

Given the heightened expression of hCG in tumors and its association with poor prognosis, the utility of strategies targeting the hormone have been explored. The anti-tumor effects of anti-hCG antibodies and anti-sense oligonucleotides have been demonstrated (Delves et al., 2007; Moulton et al., 2002). A recombinant chimeric
antibody against hCG (cPiPP) coupled to curcumin has been shown to be toxic to hCG-expressing tumor cells (Vyas et al., 2009).

An anti-tumor vaccine engineered to optimize the immune response towards hCG has been reported (Iles et al., 2010). Given the criticality of hCG to pregnancy, an anti-hCG vaccine has also been tested as an anti-fertility measure; βhCG coupled to TT underwent Phase I clinical trials in India, Finland and three other countries in 1999 (Talwar, 1999), and a βhCG-based vaccine later demonstrated efficacy in the prevention of pregnancy in women. The vaccine was safe and reversible (Talwar, 1997; Talwar et al., 1997; Pal and Singh, 2001; Singh et al., 1998). An anti-hCG vaccine also originally designed envisaged for fertility control comprises the COOH-terminal 37 amino acid peptide of hCG coupled to diphtheria toxoid (Triozzi et al., 1997). It has shown anti-tumor efficacy in patients of pancreatic and colorectal cancer; a high anti-hCG titre response to the vaccine was associated with improved survival (Moulton et al., 2002). Celldex Therapeutics (Needham, MA, USA) has developed an anti-hCGβ cancer vaccine based on their patented technology for targeting antigen presenting cells (APC) – (Keler et al., 2007). Termed CDX-1307, this vaccine completed Phase 1 development and is being investigated as a treatment for colorectal, pancreatic, bladder, ovarian and breast cancer. A Phase 2 study in patients with invasive bladder cancer began in 2010; unlike in the study by Moulton et al, 2002, inclusion was restricted to patients whose tumors expressed βhCG.

**Immunization with mycobacteria in cancer and other diseases**

Bacteria such as Bacillus Calmette Guerin (BCG), Corynebacterium parvum and streptococcal preparations have been used as adjuvants. BCG or its component cell wall skeleton (BCG-CWS) has been administered as adjuvant therapy in patients with colon or lung cancer (Yamamura et al, 1979; Hanna et al, 2001), whereas live BCG has been reported to be effective in the treatment of bladder tumors (Malmstrom et al, 1999). BCG-CWS (along with monophosphoryl lipid A) has been employed as an adjuvant for a Plasmodium falciparum circumsporozoite protein vaccine in humans, increasing immunogenicity over that observed with alum (Rickman et al, 1991). An immunotherapeutic effect of M.vaccæe administration was observed in patients with melanoma, advanced carcinoma of prostate, lung cancer and renal cell carcinoma (Hrouda et al, 1998; Stanford et al, 2008; Patel et al, 2008). The effect of the
administration of non-viable *M. smegmatis* was examined in resected lung carcinoma patients (Decroix et al, 1984). *M.w.*, the organism employed in the current studies, is a non-pathogenic strain sharing a number of B and T cell epitopes with *M.leprae* and *M. tuberculosi*s and has established efficacy as adjunct therapy in the treatment of multi-drug resistant leprosy (Ganju et al, 1991). Potential benefits have also been demonstrated in tuberculosis, HIV infection, psoriasis and asthma (Kharkar, 2002). *M.w.* administered to terminally ill, advanced cancer patients led to tumor regression, as well as improvement in clinical responses and quality of life (Dave et al., 2005; Sur and Dastidar, 2003).
**Animals and Cell Lines**

Inbred C57BL/6 and NIH nude mice were obtained from the Animal facility of the National Institute of Immunology (NII), New Delhi, where animals are bred and housed according to guidelines of the National Institute of Health. All animal procedures were performed with the approval of the Institutional Animal Ethics Committee of the NII. COLO 205 (CCL222, human colorectal carcinoma), ChaGo, (HTB168, human non-small cell lung carcinoma) and LLC (CRL1642, mouse Lewis lung carcinoma) were obtained from ATCC and maintained in culture as adherent monolayers in RPMI medium (COLO 205 and ChaGo) or DMEM (LLC), supplemented with 10% FCS.

**Administration of anti-hCG antibodies in tumor implanted mice**

Female NIH nude mice (n = 18), 6-8 weeks old, were injected subcutaneously with varying numbers of cancer (COLO 205, ChaGo, CCL-253 and LLC) cells, after which tumor growth was assessed. A dose of $10^4$ cells was shown to be optimum. Mice were divided into 3 groups: control (or untreated), anti-hCG antiserum treated and non-immune serum treated. Treatment was initiated on the day of cell implantation; 20 µg of antibody equivalent was administered every four days and tumor volumes were periodically measured.

**Immunization**

Female inbred C57BL/6 mice, 6-8 weeks old, were injected subcutaneously with $10^4$ syngeneic LLC cells. Control animals received no treatment. Prior to immunization, βhCG-TT conjugate was adsorbed on alum Groups of animals (n = 10-12) received three intramuscular injections (100 µl each) at an interval of four weeks. Alum (Alhydrogel Superfos, Denmark) adsorbed βhCG-TT conjugate (1 µg βhCG equivalent) was injected with or without autoclaved $10^7$ Mycobacterium w (Mw). Two experimental conditions were employed. In the first, tumor implantation and the respective immunizations were initiated on the same day. In the second, tumor implantation was initiated one week after completing the immunizations to assess the effects of pre-existing immune activation (Figure 5.1). Tumor volumes were measured
every third day and blood samples were collected at monthly intervals. Sera were diluted appropriately and stored at -70°C until assayed by radioimmunoassay (RIA).

**Determination of antibody titers**
Anti-hCG antibody titres expressed as hCG binding capacity were determined by a direct binding RIA as described (Singh et al., 1989). hCG was iodinated (sp.act. 40 to 60 μCi/mg) by the iodogen method. The assay protocol consisted of 100 μl of diluted antiserum; 100 μl of ¹²⁵I-hCG (15,000-20000 dpm), 100 μl of 20% horse serum and 200 μl of assay buffer (50 mM PBS with 0.1% BSA and 0.1% sodium azide, pH 7.2). After incubation at 4°C for 48 h, the antibody bound fraction was precipitated by adding 500 μl PEG 8000 (12.5% final concentration) and centrifugation at 1500 x g for 20 minutes. The pellet (radioactivity) was counted in a multi-g-counter (LKB 1260). After correcting for non-specific binding, hCG binding capacity was calculated at a point(s) where proportionality was obtained between antiserum dilutions and iodinated hCG binding.

**Statistical analysis**
Statistical analysis of data was carried out on log transformed values of antibody titres. The significance level was calculated by the Student's unpaired t-test, and considered significant if p<0.05.
Immunization schedule

Figure 5.1

Figure 5.1: Immunization schedule for active immunization studies.
**Effects of anti-hCG antibodies on tumor growth in nude mice**

Whether anti-hCG antibodies could affect the rate of tumor growth in nude mice was evaluated. Studies were initially carried out to arrive at an appropriate cell number for injection (Figure 32); a dose of $10^4$ cells was chosen for subsequent studies. The passive administration of anti-hCG antibodies significantly reduced the rate of growth of COLO 205 (Figure 33A); ChaGo (Figure 33B) and LLC (Figure 33C) induced tumors. Tumors in animals administered non-immune serum, on the other hand, grew at rates comparable to those in control animals which received no treatment (Figure 34A-C). Similar inhibitory effects of anti-hCG antiserum was observed on the growth of CCL-253 induced tumor as well (Figure 35A, B), which translated into enhanced animal survival (Figure 35C).

**Effect of Immunization on the Growth of Tumor**

The effects of active immunization with βhCG-TT, M.w or βhCG-TT + M.w on the growth of LLC induced tumors in C57BL/6 mice were assessed. When immunization and tumor implantation were simultaneously initiated, animals receiving βhCG-TT or M.w demonstrated significant decreases in tumour volume; co-immunization of βhCG-TT and M.w appeared to provide additional benefits. Figure 4 depicts results on individual animals in the three groups. Figure 5A represents average tumor volumes and tumor incidence at Day 91 post immunization in the three groups, one again demonstrating the synergistic benefits of βhCG-TT + M.w. co-immunization. These results were further substantiated by survival data. While all animals in control group succumbed by week 22, 18% of βhCG-TT immunized mice and 33% of M.w injected mice survived till this time. Significantly, 64% of the animals co-immunized with βhCG-TT and M.w survived (Figure 36B).

Pre-immunization and subsequent tumor engraftment appeared to provide similar tumor inhibitory effects of immunization; in these animals too, co-immunization with βhCG-TT and M.w. was clearly beneficial, in terms of the number of animals expressing tumors, mean tumor volumes as well as the number of animals surviving till week 27, the last point observation (Figure 37A, B). Anti-hCG antibody titers in this group of animals were significantly higher than in animals receiving βhCG-TT alone (Figure 38).
Figure 32: Assessment of tumor volumes subsequent to implantation of varying numbers of tumor cells in nude mice. (A) COLO 205, (B) LLC and (C) ChaGo
Figure 33: Effects of the passive administration of anti-hCG antibodies and normal serum on tumor volumes. (A) COLO 205, (B) ChaGo, (C) LLC.
Figure 34: Effects of the passive administration of anti-hCG antibodies and normal serum in nude mice (n=3) implanted with CCL-253 cells. (A) Tumor volumes in individual mice. (B) Tumor volumes at Day 42 (mean ± SEM). *p<0.05 vs normal serum. (C) Survival curves.
Figure 35: Growth of tumors in C57BL/6 mice implanted with LLC cells. The effects of co-immunization with βhCG-TT, M.w. or βhCG-TT + M.w. are shown in individual mice.
Figure 36: Growth of tumors in C57BL/6 mice implanted with LLC cells. The effects of co-immunization with βhCG-TT, M.w. or βhCG-TT + M.w. are shown. (A) Average tumor volumes and incidence rates at Day 91 post-implantation. Each symbol represents an individual animal. (B) Survival graph for animals implanted with LLC cells and co-immunized with βhCG-TT, M.w. or βhCG-TT + M.w., as indicated. Numbers in boxes indicate percentage survival at 22 weeks.
Figure 37: Growth of tumors in C57BL/6 mice implanted with LLC cells. The effects of pre-immunization with βhCG-TT, M.w. or βhCG-TT + M.w. are shown. (A) Average tumor volumes and incidence rates at Day 91 post-implantation. Each symbol represents an individual animal. (B) Survival graph for animals implanted with LLC cells and co-immunized with βhCG-TT, M.w. or βhCG-TT + M.w., as indicated.
Figure 38: Anti-hCG titers in mice immunized with βhCG-TT with or without M.w. on 63 day. n=10


References


