Hormone (or more frequently its individual subunits) is also synthesized by the cancers of different lineages. Interestingly, in some instances, its presence has been associated with poor radio- and chemo-resistance and poor patient prognosis. The reasons behind these associations are not clear at present. Some studies suggest that the hormonal subunits may exert autocrine effects. Therapeutic anti-hCG vaccination is now being considered, possibly as an adjunct to chemotherapy.

Colorectal cancer is associated with significant morbidity and mortality. In the present study, we have assessed the ability of anti-hCG antibodies to bind to the cell surface and internal moieties of colorectal tumour cells (CCL-253). The recognition of cellular antigens is also analyzed by the Western blot. The anti-tumor activity of anti-hCG antibodies has been evaluated in vitro (both in the absence and presence of exogenously-supplied complement) and in vivo (in tumor implantation studies in nude mice).

Materials and Methods

Cell lines and tissue culture

CCL-253 (colorectal cancer) cells were obtained from American Type Culture Collection (ATCC). The cell line was maintained in culture as an adherent monolayer in RPMI medium supplemented with 10% foetal calf serum (FSC).
Reactivity of anti-hCG antiserum with tumor cells

**Immunofluorescence analysis**

CCL-253 cells were plated at a density of $5 \times 10^5$ cells/well. Cells were permeabilized by brief incubation with 0.05% Triton X-100 in chilled methanol. Subsequent to washes with phosphate-buffered saline (PBS), non-specific binding sites were blocked by incubation for 1 h at room temperature (RT) with PBS containing 2% bovine serum albumin (BSA). Incubation with goat anti-hCG antiserum or non-immune serum (both diluted 1:500) was carried out for 2 h at RT. Cells were then incubated with 1:100 diluted secondary antibody (Jackson ImmunoResearch) for 1 h at RT. Samples were analyzed on a Nikon fluorescence microscope.

**Western blot analysis**

CCL-253 cells were harvested and cell lysates prepared. Protein concentrations were estimated by the BCA method (Pierce). After gel electrophoresis, resolved moieties were transferred on to nitrocellulose membranes (mdi, Ambala, India). Non-specific sites on the nitrocellulose membrane were blocked by incubation with Tris buffered saline (TBS) containing 3% BSA for 2 h at RT. The membrane was then washed six-times with TBS containing 0.05% Tween-20 (TBST); washes involved 10 min incubations at RT with gentle rocking. Primary antibody (goat anti-hCG antiserum or normal serum diluted 1:500 in TBS containing 0.05% Tween-20 and 1% BSA) was added to membrane strips and incubated for 2 h at RT. Strips were washed extensively with TBST to remove unbound antibodies. A rabbit anti-goat horse radish peroxidise (HRP) conjugate (diluted 1: 8000 in TBS containing 0.05% Tween-20 and 1% BSA) was added to the strips and incubated for 1 h at RT. After further washes, enzyme activity was visualized by chemiluminescence (ECL, Biological Industries, Israel). Briefly, equal volumes of the two supplied reagents were mixed and the solution was incubated with nitrocellulose membrane for 3-5 min in the dark. The reagent was then drained and membrane was exposed to X-ray film (Kodak) which was then developed.

**Effect of anti-hCG antiserum on tumor cell growth**

**In vitro**

CCL-253 cells ($3 \times 10^4$) were seeded in wells of a 96-well plate and overnight incubated under standard cell culture conditions. Cells were then cultured in serum-free medium (BioWhittaker, Switzerland) or serum-free medium containing anti-hCG antiserum or normal serum with or without 14% complement and incubated further for 24 h under standard cell culture conditions. Thereafter, cells were washed with PBS and MTT reagent (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; Sigma, USA) added and incubated for 5 h. The stop solution (50% DMSO in 20% SDS) was then added and incubated for 1 h. Cell proliferation/viability was determined colorimetrically by measuring absorbance at 550 nm.

**In vivo**

For all procedures in animals, prior approvals were obtained from the Institutional Animal Ethics Committee. The 6-8 weeks old female NIH nude mice were injected subcutaneously with $10^7$ CCL-253 cells in the dorsal area. Mice were divided into two groups: one group received anti-hCG antiserum and the other normal serum. Treatment was initiated on the day of implantation; 20 µg of antibody equivalent was administered every four days and tumor volumes were periodically measured. Tumor size was determined with callipers at weekly intervals and tumor volumes calculated according to the following formula: $1/2 \times l \times w^2$, where $l$ represents the longest axis of tumor and $w$, the axis perpendicular to $l$.

**Results**

Reactivity of anti-hCG antiserum towards tumor cells

In indirect immunofluorescence microscopy, anti-hCG antiserum demonstrated cell surface as well as cytoplasmic reactivity towards CCL-253 cells. On the other hand, normal serum was non-reactive towards both cell surface and cytoplasmic moieties (Fig. 1).

Western blot analysis of whole-cell lysates derived from CCL-253 cells showed reactivity of anti-hCG antiserum towards multiple moieties, demonstrating the putative recognition of cross-reactive moieties; normal serum was devoid of such reactivity (Fig. 2).

**Anti-tumor effect of anti-hCG antiserum in vitro**

Upon incubation of CCL-253 cells with anti-hCG antiserum, a significant inhibitory effect on growth was observed, while no such effect was observed with normal serum (Fig. 3). The inhibitory effect of anti-hCG anti-serum was dose-dependent (data not shown). Addition of complement along with anti-hCG antiserum significantly enhanced inhibitory effects on cell growth compared with either complement or anti-hCG antiserum added individually. No such effect in excess of that observed with complement alone was noticed on addition of complement to normal serum (Fig. 4).
Anti-tumor effect of anti-hCG antiserum *in vivo*

The growth inhibitory effect of anti-hCG antiserum was investigated upon tumor xenografts in nude mice. CCL-253 cells were subcutaneously injected into nude mice which were then treated with anti-hCG serum or normal serum. Tumor volumes in mice administered anti-hCG antiserum were significantly lower compared with those in mice treated with normal serum (Fig. 5A, B). In addition, while all mice treated with anti-hCG antiserum survived till day 120 (the last point of observation), mice treated with normal serum were dead by this time (Fig. 5C).
Fig. 5—Effect of 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; and (C): Survival curves]

Discussion

hCG was believed to be a pregnancy-specific hormone, but is now known to be secreted by a variety of cancers\textsuperscript{8-12} and in some instances, there is evidence that the hormone or individual subunits can act as a growth factor. The objective of this study was to demonstrate the anti-tumor effects of anti-hCG antiserum both \textit{in vitro} and \textit{in vivo}. A cell line derived from human colorectal cancer was chosen for the study. Colorectal cancer represents a significant human disease burden; it is the third most common cancer and a leading cause of cancer-related mortality in both men and women\textsuperscript{16}.

In present study, the presence of moieties reactive to anti-hCG antibodies was demonstrated on the cell surface and in the cytoplasm of CCL-253 cells. Interestingly, anti-hCG antiserum was reactive towards multiple moieties on Western blot analysis, an indication of the existence of cross-reactive epitopes. The identity and properties of these moieties is of obvious physiological relevance and is a matter of current investigation.

Fc-dependent activities like antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) can contribute significantly to the cytotoxic action of antibodies\textsuperscript{17}, and several of the antibodies approved for the treatment of cancer in humans are believed to act via these mechanisms\textsuperscript{18}. Interestingly, in the present study, anti-hCG antibodies inhibited the growth of tumor cell line \textit{in vitro} to a significant extent, even in the absence of complement. Both subunits of hCG contain a cystine knot, a structural motif found in some growth factors\textsuperscript{19} and may, therefore, be relevant to its putative growth-promoting effects. Possibly, anti-hCG antibodies sequestered the hCG (or its subunits) secreted by the tumor cells causing the loss in viability, since cells were deprived of the autocrine growth-promoting effects of the hormone. Alternatively, binding of antibodies to the cell surface may transduce a death signal into the cell. Efforts are underway to distinguish between the two possibilities. Regardless of the mechanism of antibody-mediated loss in viability, the addition of complement had additive effects.

The anti-tumor effect of anti-hCG antibodies was apparent in a xenograft nude mouse model as well; though not completely abolished, the growth of CCL-253 cells was substantially inhibited. Possibly because of the reduced tumor burden, all treated animals were alive till the end of the experiment, a time-point at which all control animals had died.

Interestingly, specific cell surface and cytoplasmic reactivity of anti-hCG antiserum was also observed towards a human ovarian cancer cell line (OVCAR-3) and anti-hCG antiserum showed a significant inhibitory effect on cellular...
growth of these cells as well (data not shown). Ovarian cancer has the highest mortality of all cancers of the female reproductive system reflecting, in part, a lack of early symptoms and ineffective ovarian cancer screening tests. It is often diagnosed at an advanced stage after the cancer has spread beyond the ovary. Thus, as with colorectal cancer, there exists a great need to develop new methods of diagnosis and treatment.

The role of hCG both as a tumor marker and as a prognostic indicator is only now being truly appreciated. These observations lend support to the premise that strategies targeting hCG$_{3-7}$, possibly in conjunction with conventional chemotherapy can be of great therapeutic benefit in the treatment of tumors.

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References