Human acute lymphoblastic leukaemia cells make human pregnancy hormone hCG and expose it on the membrane: A case for using recombinant antibody against hCG for selective delivery of drugs and/or radiations

Rafi Shiraz Kabeer[1], Rahul Pal[2] and G. P. Talwar[1,*]

[1]Talwar Reasearch Foundation, E-8 Neb Valley, Neb Sarai, New Delhi 110 068, India

[2]Immunoendocrinology Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110 067, India

The binding of a humanized chimeric (cPiPP) recombinant antibody (human IgG_1, kappa linked to mouse variable regions) of high affinity (Ka 3×10^{10} M^{-1}) and high specificity for human chorionic gonadotrophin (hCG) with MOLT-4 cells, an acute lymphoblastic leukaemia cell line derived from a patient in relapse, has been studied. The antibody binds on the membranes of the viable tumour cells as seen by flow cytometry. Biologically active pure hCG competes with the binding. No binding is seen with an irrelevant antibody directed against an epitope on androgen independent prostate cancer cell line (DU145). Studies on permeabilized cells show that these cells synthesize both alpha and beta-hCG. Peripheral blood mononuclear cells of healthy individuals do not bind with cPiPP. The potential of this antibody for imaging and selective delivery of radiations and/or drugs to tumour cells is suggested.

Keywords: Chimeric anti-hCG antibody, ectopic expression of hCG, leukaemia cell line, membrane localization.

RELAPSE after multidrug chemotherapy is a frequent occurrence in cancers. At this stage the tumour invariably spreads to other tissues, besides becoming resilient to primary chemotherapeutic drugs. Radiotherapy takes its own toll. Search has been going on for several years to develop strategies for treatment of cancers at metastasis stage with approaches that could focus on the tumour cells without undue toxicity to other tissues. Amongst the emerging approaches are the use of antibodies directed at epitopes/markers primarily localized on cancer cells and completely absent or present at low concentrations on normal cells.

We report here the unexpected presence of human chorionic gonadotrophin (hCG) on membranes of a human T lymphoblastic leukaemia cell line derived from a patient in relapse, who had received prior multidrug chemotherapy. The cells from this patient were developed by ATCC as a cell line (CRL-1582) with the designation MOLT-4. MOLT-4 does not produce immunoglobulins nor has Epstein-Barr virus. MOLT-4 cells have variable expression of various CD antigens [CD1 (49%), CD2 (35%), CD3 A (26%), CD3 B (33%), CD5(72%), CD6(22%) and CD7(77%)]. The interesting feature of the observation to be described here, is the presence of hCG on almost all cells. The cells not only synthesize hCG, as is evident from studies on permeabilized cells, but also carry it on their membranes. An antibody of high specificity raised against the beta subunit of hCG has been humanized and expressed as a recombinant protein in plants[1]; it binds to about 95% of the tumour cells.

Materials and methods

Cell cultures

MOLT-4 cells (ATCC CRL-1582) were grown in a humidified incubator at 37°C under 5% CO_2 and 95% air in RPMI1640 medium (Gibco) with 10% foetal calf serum (Hyclone) and antibiotic–antimycotic (penicillin 100 µg/ml and streptomycin 0.25 g/ml; Gibco). The cells were subcultured before they reached more then 70% confluency. A haemocytometer was used to count the cells and viability was determined by Trypan blue exclusion. An additional criterion employed for checking the viability was with propidium iodide (PI).

Antibodies

cPiPP is a chimeric recombinant antibody engineered from a mouse monoclonal raised against beta-hCG[2]. The antibody is a recombinant protein having human IgG_1 as constant heavy chain and human kappa as constant light chain fused with variable chains of mouse monoclonal antibody[1]. The antibody is expressed at high yield in tobacco leaves, as described previously[3]. It was purified by protein A affinity

*For correspondence. (e-mail: talwar37@rediffmail.com)

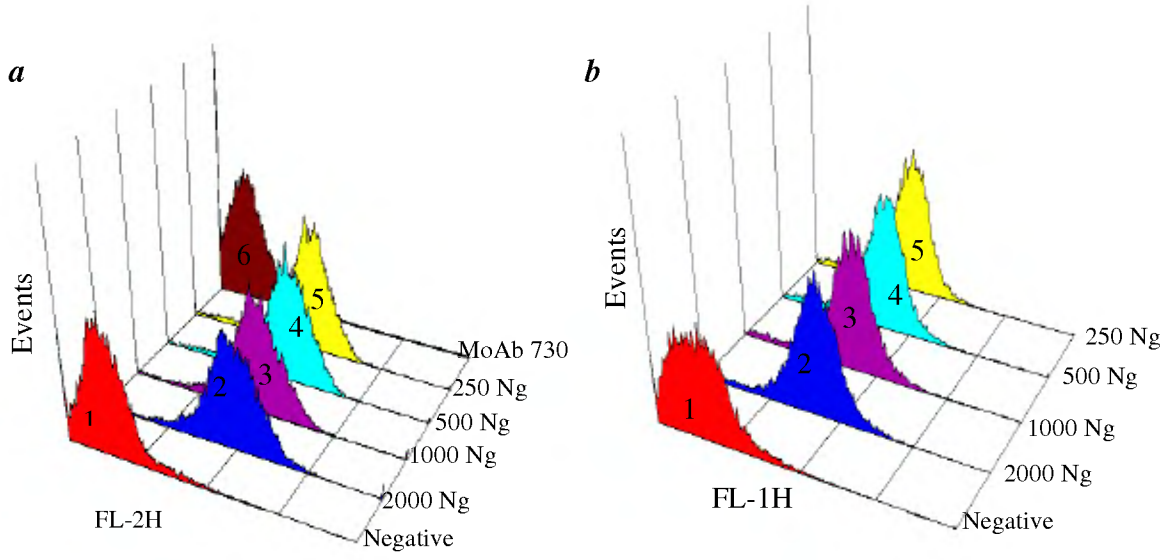


Figure 1. Reactivity of (***a***) anti-alpha-hCG (P_{22376}) and (***b***) anti-beta-hCG (cPiPP) antibodies to MOLT-4 cells. FACS analysis was carried out at antibody concentrations. In each case, 80–91% of cells show binding with these antibodies. Histogram 1 in both (***a***) and (***b***) shows fluorescence of cells without antibody. A non-hCG-reactive monoclonal antibody (MoAb 730) demonstrated a lack of recognition (Histogram 6 in (***a***)).

chromatography. The association constant of the antibody for hCG is 3×10^{10} M^{-1}. The antibody is specific to hCG and does not recognize human TSH and human FSH both sharing the same alpha chain with hCG. It has < 5% cross reactivity with human LH[2].

P_{22376} is a mouse monoclonal raised against alpha-hCG[4]. It was purified by protein A affinity chromatography from ascites raised in syngenic Balb/c mice.

Monoclonal antibody 730 (MoAb 730) was raised against DU145 cells which are androgen independent prostate carcinoma[5]. This antibody was used as a negative control to rule out any non-specific binding of the anti-hCG antibodies to the MOLT-4 cells.

A humanized chimeric antibody against LH-RH in which human IgG_1, kappa is fused with mouse variable region, was employed as antibody isotype control for cPiPP.

Flow cytometry

Fluorescene isothiocyanate (FITC) IgG_1-labelled goat anti-human IgG (Jackson Immuno Research) and phycoerythrin (PE)-labelled goat anti-mouse IgG (H + L) (Jackson Immuno Research) were used in flow cytometric analyses to detect the binding of cPiPP and P_{22376} respectively.

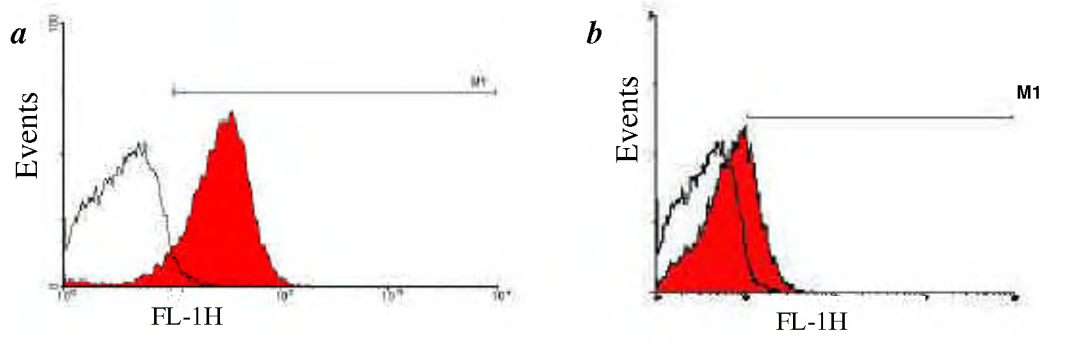
Two million cells were suspended in 1 ml FACS buffer (10 mM PBS, 1% BSA, 0.2% sodium azide) and 50 µl of the suspension was aliquoted per well per reaction, in a 96-well round-bottom plate (Nunc). The cells were incubated with 2 µg cPiPP taken in 50 µl FACS-buffer for 1 h at 4°C. The cells were washed three times with FACS buffer, each time centrifuging at 500 *g* for 2 min at 4°C. FITC-conjugated goat anti-human IgG at a dilution of 1 : 50 was used to detect the binding of cPiPP to MOLT-4 cells. For detection of binding of the mouse monoclonal antibody P_{22376}, PE-labelled goat anti-mouse IgG at 1 : 50 dilution was used. For flow cytometric analysis, 5000 or 10,000 cells were analysed with BD LSR (Becton–Dickinson) instrument using WinMdi software (version 2.7).

Detection of intracellular synthesis of beta/alpha-hCG was done by flow cytometry using permeabilized cancer cells. For permeabilization, cells were incubated in chilled solution of methanol containing 0.01% Triton-X placed on ice for 1 min. After resuspension of cells in FACS buffer, staining was carried out as before.

Competition experiments with hCG (10,000 IU/mg) were conducted as follows: an aliquot of cPiPP was pre-incubated with 10 µg hCG for 1 h at 37°C. The binding capacity of the antibody was determined against a control in which the antibody was incubated under similar conditions with an equivalent volume of 10 mM Phosphate Buffer Saline (PBS). FACS analysis was undertaken to asses a decrease in cellular staining.

Immunohistochemistry

Two million cells were suspended in 1 ml FACS buffer (10 mM PBS, 1% BSA, 0.2% sodium azide) and 50 µl of the suspension was aliquoted per well per reaction, in a 96-well-round bottom plate (Nunc). The cells were permeabilized by incubating in chilled solution of methanol containing 0.01% Triton-X placed on ice for 1 min. After resuspension, the cells were incubated with 2 µg cPiPP taken in 50 µl FACS buffer for 1 h at 4°C. The cells were washed three times with FACS buffer, each time centrifuging at 500 *g* for 2 min at 4°C. Horse Radish Peroxidase (HRP) conjugated goat anti-human IgG (Jackson Immuno Research)



Multiple Document Interface for Flow Cytometry
WinMDI Version 2.7 - Windows 3.95/DOS 5.0
Total Events 10000 Gated Events 10000 100.00%
System: Log Parameter Means: Geometric

Param name	M	Low,	High	Events	%Total	%Gated
FL1-H	0	0,1023	10000	100.00	100.00	26.28
	1	265,1023	9077	90.77	90.77	30.87

Multiple Document Interface for Flow Cytometry
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Param name	M	Low,	High	Events	%Total	%Gated
FL1-H	0	0,	1023	10000	100.00	100.00
	1	258,	1023	2992	29.92	29.92

Figure 2. Competition experiments with authentic hCG. cPiPP was preincubated with 10 µg of purified hCG and binding of the antibody with and without incubation with hCG was determined by flow cytometry. The number of cells binding with the antibody declined from (*a*) 90.77% to (*b*) 29.9% on scavenging of hCG-binding sites on the antibody by hCG.

at a dilution of 1 : 400 was used as a second antibody to detect the binding of cPiPP to MOLT-4 cells and the cells were incubated in it for 1 h. After washing three times with FACS buffer, 2,4-diamino benzidine (DAB) was added as substrate followed by an incubation period of 15 min. The reaction was stopped by washing the cells with PBS. Cells were then examined under microscope for binding of the antibody.

Results

Figure 1 shows the binding of cPiPP and P_{22376} to the surface of MOLT-4 cells, as observed by FACS analyses. MOLT-4 cells employed for FACS analyses were more than 95% viable as determined by Trypan blue exclusion and lack of PI uptake. A non-hCG reactive mouse monoclonal (MoAb 730) differing in antigen binding regions and employed as a negative control demonstrated the lack of such binding with the tumour cells. As MoAb 730 is a monoclonal of mouse origin and shows lack of binding to MOLT-4 cells, it can be deduced that no nonspecific adhesion of the light and heavy chains takes place with the tumour cells.

In order to determine whether the binding of cPiPP was indeed to hCG exposed on the membranes of cells, competition experiments with authentic purified hCG hormone were conducted. As shown in Figure 2, with the saturation of specific antigen-binding regions by hCG, lower binding capacity of the antibody was available for the hormone present on the membrane of tumour cells. Whereas at 500 ng of antibody, 90% of MOLT-4 cells were binding the antibody, on competition with hCG, the specific antigen-binding capacity of the antibody was diminished; and only 30% of the cells bound with the residual antibody.

Further evidence for cancer cells synthesizing both alpha- and beta-hCG was obtained by intracellular staining of the cells with the two antibodies reacting specifically with alpha- and beta-hCG. The cells were permeabilized to enable the entry of alpha- and beta-hCG reactive antibodies into the cells, so that these could attach to the protein made by the cells and then be processed for FACS analysis. Figure 3 *a* and *b* show clearly that majority of the cells were reactive with both the antibodies and binding appeared to be more than that observed on the membrane of cells.

In view of reports[6] that some cancer cells such as ChaGo (a non small cell lung cancer) express alpha-hCG and others express beta-hCG, it was of interest to determine whether diversity existed in the expression of either alpha- or beta-hCG in MOLT-4 cells. Double colour FACS analyses of MOLT-4 cells was carried out, taking advantage of the fact that cPiPP has human Fc region and can be detected by FITC-conjugated goat anti-human IgG, and P_{22376} being a mouse monoclonal, could be detected by PE-labelled goat anti-mouse IgG (Figure 4). We observed that more than 95% cells recognized both antibodies, indicating the presence of both subunits on the same cell.

Further confirmation of the binding of cPiPP was obtained by immunohistochemistry. MOLT-4 cells incubated with cPiPP bound with MOLT-4 cells as revealed by immuno-peroxidase staining (Figure 5 *b*). Incubation with another chimeric humanized antibody against LH-RH (cLH-RH), which bears the same isotype as chimeric cPiPP for heavy and light chain constant regions, was devoid of such binding (Figure 5 *c*).

In view of the observation that the cancerous cells of this T-cell lymphoblastic leukaemia carry hCG on their membrane to which cPiPP binds, an obvious use of such antibody would be to employ it for imaging and delivery of radiations

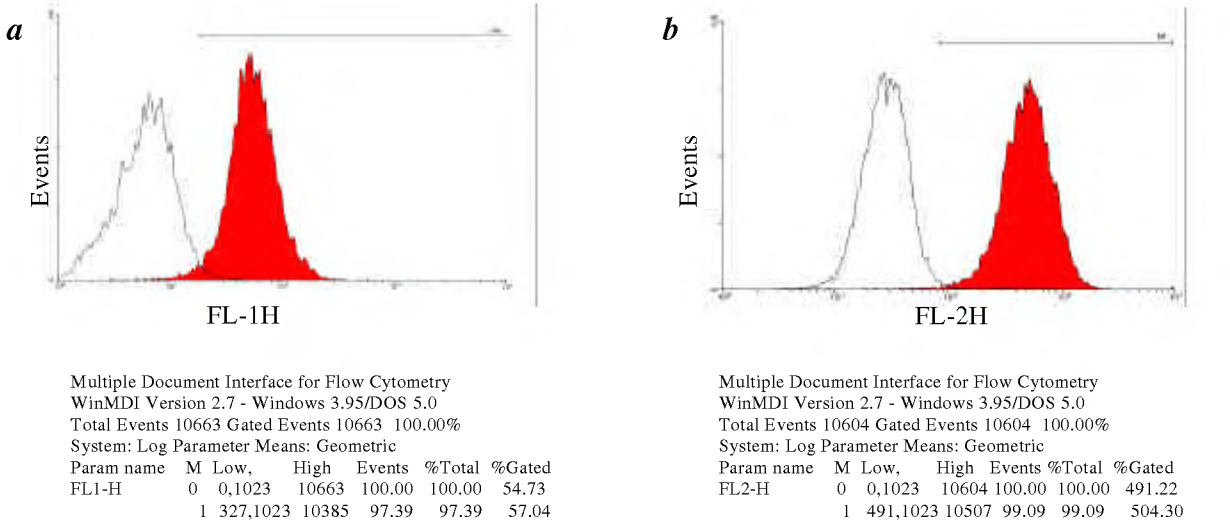

Figure 3. Intracellular production of hCG by MOLT-4 cells. Open histograms in both (***a***) and (***b***) are controls. Filled histograms represent data obtained on incubation of permeabilized MOLT-4 with cPiPP (***a***) and P_{22376} (***b***). Permeabilized cells show 97% binding with (***a***) cPiPP and 99% with (***b***) P_{22376}, indicating production of hCG within MOLT-4 cells.

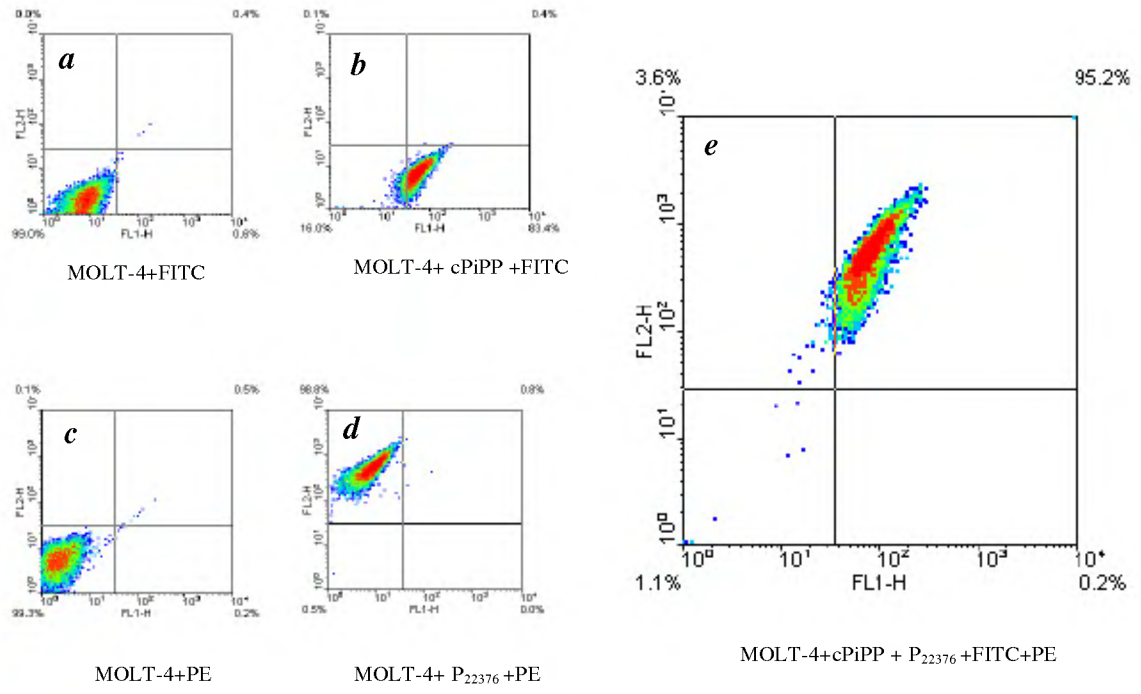

Figure 4. Dot-plot profiles of binding of anti-hCG antibodies to MOLT-4 cells. ***a***, ***b***, Fluorescence profile of cells incubated with (***a***) detection antibody (anti-human FITC) alone and (***b***) anti-beta-hCG chimeric antibody (cPiPP) followed by incubation with detection antibody (anti-human FITC). More than 83% cells move in the lower right (LR) quadrant indicating binding with cPiPP compared to control. ***c***, ***d***, Fluorescence profile of cells incubated with (***c***) detection antibody (anti-mouse PE) alone and (***d***) anti-alpha-hCG antibody (P_{22376}) followed by incubation with detection antibody (anti-mouse PE). More than 95% cells show binding to anti-alpha-hCG antibody compared to control, as shown in the upper left (UL) quadrant. ***e***, Fluorescence profile of cells incubated with both cPiPP and P_{22376} followed by incubation with the two detection antibodies. About 95% of the cells bound with both these antibodies on the surface, as seen in the upper right (UR) quadrant of the profile, indicating that both the antibodies recognize hCG present on the surface of the same cell.

and drugs to the tumour cells. It was therefore necessary to determine whether this antibody discriminates between cancer cells and the normal healthy cells of the same lineage. Thus, binding of cPiPP with Peripheral Blood Mononuclear Cells (PBMC) of healthy normal people was investigated. cPiPP did not bind with PBMC, demonstrating thereby the ability of cPiPP to bind selectively to cancer cells, sparing normal healthy leukocytes (Figure 6).

This experiment suggests the ability of cPiPP to distinguish between healthy and cancer cells for localization.

Discussion

hCG is primarily considered as a pregnancy hormone and its secretion in urine or blood is taken as a confirmed criterion for diagnosis of pregnancy. Non-pregnant healthy women (and men) do not secrete this hormone in detectable amounts. Ectopic synthesis of alpha-, beta- or both subunits of hCG has been reported in lung cancer[7,8], urothelial cancers[9], colon adenocarcinoma[10,11], pancreartic carcinoma[12,13] liver malignancies, neuroendocrine tumours[14] and in patients with poorly differentiated carcinomas[15]. Syrigos *et al.*[16] reported that 40% of pancreatic exocrine tumours produce beta-hCG and its production is correlated with adverse survival. The survival time for beta-hCG positive colorectal cancers was significantly shorter than beta-hCG negative cancer patients[17].

The data communicated here show the synthesis of both alpha- and beta-hCG by MOLT-4, T-lymphoblastic leukaemia cells. While these are synthesized and are detectable intracellularly, their presence on membranes of tumour cells is demonstrated by the binding of both anti-alpha and anti-beta antibodies on the surface of live cells, as seen by flow cytometry. This implies that the hormonal subunits are most likely in associated state, as hCG is secreted and picked up by receptors for this hormone on membranes of tumour cells. Over 90% of cells bind to cPiPP. The binding of monoclonal against alpha-hCG with the same cells is indicative of the receptor on these cells for the full native hormone, hCG. The binding is competed by highly purified biologically active hCG, and an irrelevant antibody such as the one directed at androgen-independent carcinoma cells does not bind with MOLT-4 cells. Furthermore, a chimeric antibody against an irrelevant antigen LH-RH does not bind with MOLT-4 cells as determined immunohistochemically (Figure 5 *c*), excluding the possibility of non-specific attachment of human IgG_1 and kappa with these cancer cells. Data in Figure 6 also clearly show the lack of reactivity of cPiPP with PBMC isolated from healthy volunteers, thereby demonstrating the selective ability of this antibody to home to the tumour cells. This antibody may

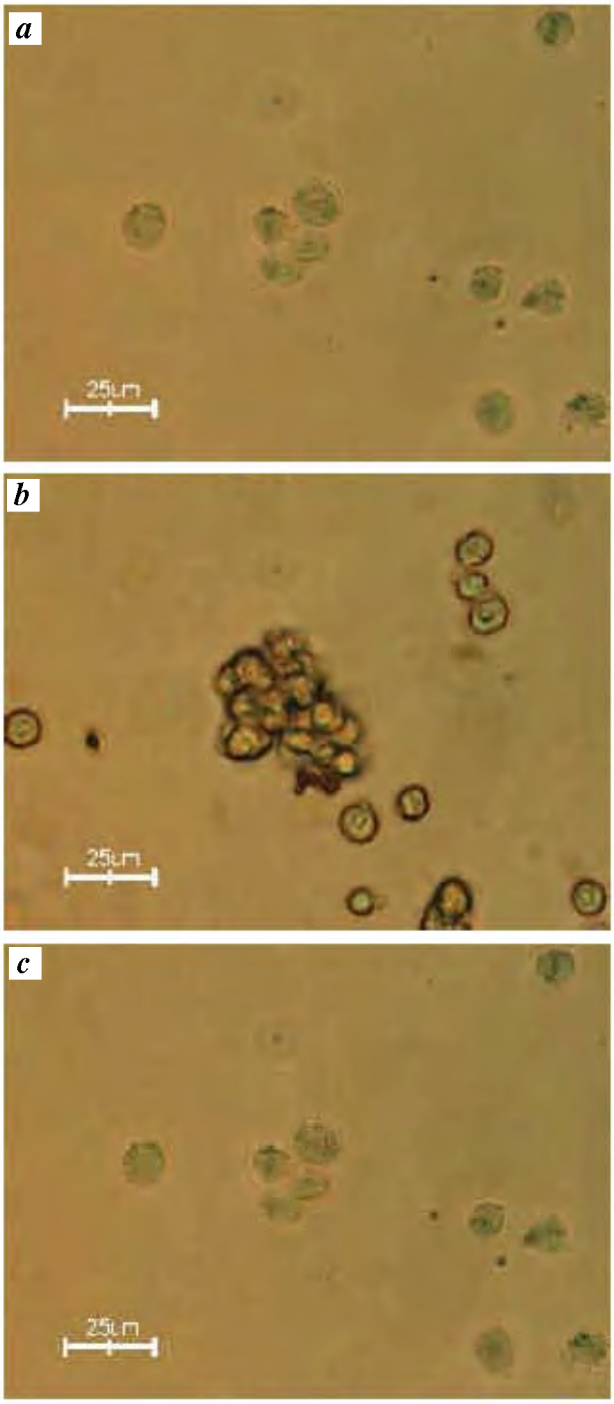

Figure 5. Immunohistochemical localization of humanized chimeric antibody against beta-hCG (cPiPP) on MOLT-4 cells. ***a***, Control without cPiPP. ***b***, With cPiPP. ***c***, With humanized chimeric antibody against LH-RH, an isotype control.

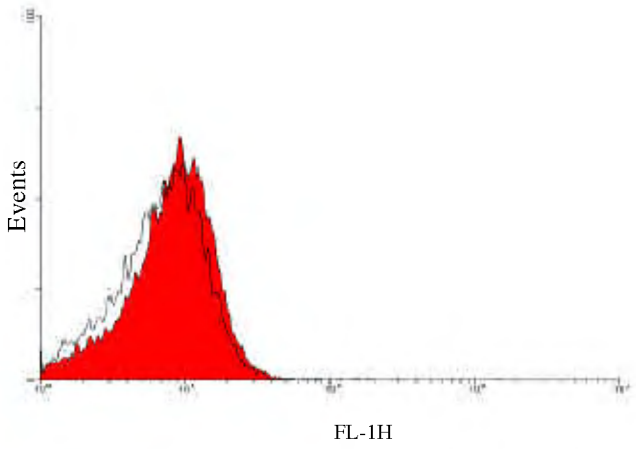

Figure 6. cPiPP antibody discriminates between cancer and normal peripheral blood mononuclear cells (PBMC) for binding. Migration profile of PBMC after incubation with cPiPP antibody. Profile of the cells overlapped the control PBMC not exposed to the antibody.

thus be useful for imaging and selective delivery of radiations and/or drugs to such tumour cells without undue toxicity to normal healthy cells. The lack of cross-reaction of anti-hCG antibodies with normal tissues is also reflected by the multicentre phase I trials on a vaccine engendering anti-hCG antibodies[18–20]. Furthermore, the continuous presence of anti-hCG antibodies in circulation in sexually active healthy women over two and a half years without any side effects, vouches for the long-term safety of anti-hCG antibodies[21,22]. Women had no obvious complaint or discomfort, experienced normal ovulatory events and had regular menstrual cycles. The epitope against which 18–86% of total circulating antibodies of immunized women were directed overlaps with the epitope recognized by the chimeric antibody used in these studies[23].

1. Kathuria, S. *et al.*, Efficacy of plant produced recombinant antibodies against hCG. *Hum. Reprod.*, 2002, **17**, 2054–2061.
2. Gupta, S. K., Ramakrishnan, S. and Talwar, G. P., Properties and characteristics of an anti-hCG monoclonal antibody. *J. Biosci.*, 1982, **4**, 105.
3. Kathuria, S., Nath, R., Pal, R., Singh, O., Fischer, R., Lohiya, N. K. and Talwar, G. P., Functional recombinant antibodies against hCG expressed in plants. *Curr. Sci.*, 2002, **82**, 1452–1457.
4. Gupta, S. K., Singh, O., Kaur, I. and Talwar, G. P., Characteristics of monoclonal anti-alpha-human chorionic gonadotropin antibody. *Indian J. Med. Res.* 1985, **81**, 281–285.
5. Talwar, G. P. *et al.*, A monoclonal antibody cytolytic to androgen independent DU145 and PC3 human prostatic carcinoma cells. *Prostate*, 2001, **46**, 207–213.
6. Kumar, S., Talwar, G. P. and Biswas, D. K., Necrosis and inhibition of growth of human lung tumour by anti-alpha-human chorionic gonadotropin antibody. *J. Natl. Cancer Inst.*, 1992, **84**, 42–47.
7. Ikura, Y., Inoue, T., Tsukuda, H., Yamamoto, T., Ueda, M. and Kobayashi, Y., Primary choriocarcinoma and human chorionic gonadotrophin-producing giant cell carcinoma of the lung: Are they independent entities? *Histopathology*, 2000, **36**, 17–25.
8. Acevedo, H. F., Krichevsky, A., Campbell-Acevedo, E. A., Galyon, J. C., Buffo, M. J. and Hartsock, R. J., Expression of membrane-associated human chorionic gonadotropin, its subunits, and fragments by cultured human cancer cells. *Cancer*, 1992, **69**, 1829–1842.
9. Iles, R. K. and Chard, T., Enhancement of ectopic beta-human chorionic gonadotrophin expression by interferon-alpha. *J. Endocrinol.*, 1989, **123**, 501–507.
10. Kiran, R. P., Visvanathan, R. and Simpson, C. G., Choriocarcinomatous metaplasia of a metachronous adenocarcinoma of the colon. *Eur. J. Surg. Oncol.*, 2001, **27**, 436–437.
11. Lundin, M. S., Nordling, M., Carpelan-Holmstrom, Louhimo, J., Alfthan, H., Stenman, U. H. and Haglund, C., A comparison of serum and tissue hCG beta as prognostic markers in colorectal cancer. *Anticancer Res.*, 2000, **20**, 4949–4951.
12. Alfthan, H., Haglund, C., Roberts, P. and Stenman U. H., Elevation of free beta subunit of human chorionic gonadotropin and core beta fragment of human chorionic gonadotropin in the serum and urine of patients with malignant pancreatic and biliary disease. *Cancer Res.*, 1992, **52**, 4628–4633.
13. Taylor, H., Heaton, N., Farrands, P., Kirkham, N. and Fletcher, M., Elevated human chorionic gonadotrophin levels in a patient with pancreatic carcinoma presenting with atesticular metastasis. *Postgrad. Med. J.*, 1990, **66**, 1073–1075.
14. Bidart, J. M., Baudin, E., Troalen, F., Bellet, D. and Schlumberger, M., Eutopic and ectopic production of glycoprotein hormones alpha and beta subunits. *Ann. Endocrinol.*, 1997, **58**, 125–128.
15. Sumi, H. *et al.*, Treatable subsets in cancer of unknown primary origin. *Jpn. J. Cancer Res.*, 2001, **92**, 704–709.
16. Syrigos, K. N. *et al.*, Beta human chorionic gonadotropin concentrations in serum of patients with pancreatic adenocarcinoma. *Gut*, 1998, **42**, 88–91.
17. Lundin, M., Nordling, S., Lundin, J., Alfthan, H., Stenman, U. H. and Haglund, C., Tissue expression of human chorionic gonadotropin beta predicts outcome in colorectal cancer: A comparison with serum expression. *Int. J. Cancer*, 2001, **95**, 18–22.
18. Kharat, I. *et al.*, Analysis of menstrual records of women immunized with anti-hCG vaccines inducing antibodies partially cross-reactive with hLH. *Contraception*, 1990, **41**, 293–299.
19. Nash, H. *et al.*, Observations on the antigenicity and clinical effects of a candidate antipregnancy vaccine: Beta-subunit of human chorionic gonadotropin linked to tetanus toxoid. *Fertil. Steril.*, 1980, **34**, 328–335.
20. Talwar, G. P. *et al.*, Phase I clinical trials with three formulations of anti-human chorionic gonadotropin vaccine. *Contraception*, 1990, **41**, 301–316.
21. Talwar, G. P. *et al.*, A vaccine that prevents pregnancy in women. *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 8532–8536.
22. Talwar, G. P. *et al.*, The HSD-hCG vaccine prevents pregnancy in women: feasibility study of a reversible safe contraceptive vaccine. *Am. J. Reprod. Immunol.*, 1997, **37**, 153–160.
23. Deshmukh, U. S., Pal, R., Talwar, G. P. and Gupta, S. K., Antibody response against epitopes on hCG mapped by monoclonal antibodies in women immunized with an anti-hCG vaccine and its implications for bioneutralization. *J. Reprod. Immunol.*, 1993, **5**, 103–117.

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