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In Vitro Selective Modulation of Cellular Glutathione by a Humanized Native Milk Protein Isolate in Normal Cells and Rat Mammary Carcinoma Model

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Abstract. *We report the in vitro selective inhibitory activity of a humanized whey protein concentrate IMMUNOCAL™ on growth of mammary carcinoma cells and Jurkat T cells in comparison to normal peripheral blood mononuclear cells. We relate this inhibitory activity to a selective depletion of intracellular gluthathione synthesis. The use of humanized whey protein concentrate as a food supplementation may have direct implication in clinical trial with adjuvant chemotherapy.*

Glutathione (GSH) accounts for more than 90% of total intracellular non-protein sulfhydryl and is critical in a variety of cellular defense functions including protection from toxic oxygen species and detoxification of various xenobiotics (1). Tumor cell GSH concentration may be among the determinant of the cytotoxicity of many chemotherapeutic agents and of radiation, and an increase in GSH concentration appears to be at least one of the mechanism of acquired drug resistance to chemotherapy (2,3,4).

Therapeutic elevation of normal cell GSH level has also been investigated as a mean to reduce the toxicity associated with a wide variety of compounds of both endogenous and exogenous origin (5).

GSH may be increased by different methods including delivery of L-Cystine, a rate limiting amino acid in GSH synthesis. This is difficult since cysteine is toxic, it is not transported efficiently into cells, and is oxidized spontaneously at neutral pH (5).

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Attempts to cancer treatment based on modulation of GSH concentration in tumor cells must take into consideration the glutathione status and the rate of GSH synthesis in these cells. It is well known that rapid GSH synthesis in tumor cells is associated with high rates of cellular proliferation (6). Depletion of tumor GSH *in vivo* decreases the rate of cellular proliferation and inhibits cancer growth. In practice it is difficult to reduce GSH sufficiently in a tumor *in vivo* without placing the normal tissue at risk.

Numerous studies have demonstrated that GSH can be differently manipulated in normal versus tumor cells line (7,8). Dependent upon the method of GSH manipulation protection could be demonstrated in normal but not in tumor cell line.

In this report we demonstrate that it is possible to selectively modulate *in vitro* GSH synthesis in normal cells compared to cancer cells with a humanized (HWPC) Whey Protein Concentrate and that this selective GSH modulation has an impact on cells proliferation.

Materials and Methods

Cells and reagents. Normal peripheral blood mononuclear cells were obtained from healthy volunteers as a normal control. Cancer cells were a MATB 13672 cell line derived from a female fisher rat mammary tumor kindly provided by Dr Gerry Batist (Lady Davis Research Institute, Montreal) and Jurkat T cells kindly provided by Dr. Bruce Mazer (Montreal Children's Hospital). Cells were grown *in vitro* in 96 wells plates for proliferation assay and in 24 wells plates for GSH determination.

MATB cells were cultured in alpha MEM (Gibco) supplemented with 10% fetal bovine serum, 1% non essential amino-acid, 2% glutamine and 2% sodium pyruvate; Jurkat T cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% glutamine, and 1% penicillin streptomycin. Cells were cultured during 72 hours then harvested for proliferation assays and GSH determination.

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Whey protein concentrate. Pathogen free Immunocal™ a humanized whey protein concentrate (HWPC), prepared in a special fashion, was supplied as a powder by IMMUNOTEC Research corporation (MONTREAL QUEBEC). It had the following characteristics: pure protein content 77% with a solubility index of 99% at pH 4.6. Protein composition as percentage of total protein measured by polyacrylamide gel electrophoresis was: betalactoglobulin 56.3%, alpha lactalbumin 22.8% serum albumin 11.1%, lactoferrin 0.7% and immunoglobulin 9.2%.

Standard bacteriological tests were negative. This powder was resuspended in RPMI 1640 (10g/100ml) and centrifuged at 30000g for 15 min at 15° C. The supernatant was filtered on a 0.2 µm filter and conserved at 4°C. Concentration between 0.1 µg/ml and 1mg/ml were used. Pure sodium caseinate was used as a negative control for proliferation and glutathione assay.

Determination of whey protein concentrate toxicity. Cell proliferation and trypan blue exclusion assays were performed after 3 days of culture in order to evaluate whey protein concentrate cytotoxicity.

Glutathione determination. Intracellular GSH concentration was measured by enzymatic assay as described (9) using niacinamide diphosphate, dithio nitrobenzene and glutathione reductase. Briefly isolated lymphocytes or tumor cells were lysed with ice cold water and 1% sulfosalicylic acid, centrifuged at 5000g for ten minutes and the supernatant collected and processed. Spectrophotometric analysis was performed for 2 minutes at 412 nm and the result expressed n nmol of GSH /10^7 cells.

Proliferation assay. Cells were plated at 50000 cells/well in a 96 wells plate and incubated for 72 hours in the presence of varying concentration of IMMUNOCAL™ After 66 hours ^{3}H-TdR added to the wells (1 µCi/ wells) for an additional 6 hours Following this, cells were harvested and incorporated radioactivity determined by scintillation counter. All studies were performed in triplicate, and results were expressed as mean +/- SEM. Statistical analysis was done using the student test with a p value considered significant if $p < 0.05$

Results

Proliferation assay. On normal peripheral blood mononuclear cells (PBMC) IMMUNOCAL™ was shown to exert a dose dependent stimulation effect on cell proliferation as measured by incorporation of radiolabelled thymidine. (Figure 1) Prolifcration of PBMC was stimulated at concentration between 100 µg/ ml and 200 µg/ml, these results are statistically significant with a p value < 0.05. However no stimulation occurred at higher dose of humanized whey protein concentrate 1 mg/ml although no cytotoxicity was seen at concentration > 500 µg/ml, control cells exposed to the same concentration of casein were not affected (data not shown).

MATB and Jurkat T cells. On the contrary when incubating cancer cells either MATB cells of Jurkat T cells at the same concentration of IMMUNOCAL™ as in normal cell assay cellular proliferation was inhibited (Figure 2 and 3); no cytotoxicity was seen at these concentration and at even higher concentration between 500 µg/ml and 1000 µg/ml cellular proliferation were markedly inhibited (p value <0.05).

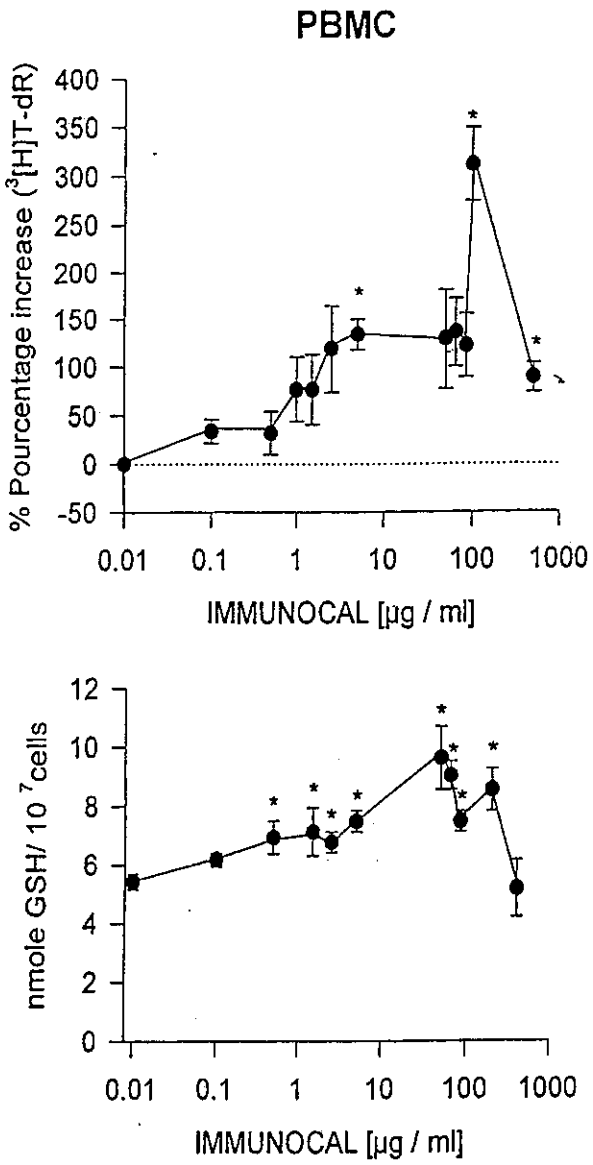

Figure 1. *Upper: Effect of Immunocal on the proliferation of peripheral blood mononuclear cells in vitro.*
Lower: Effect of Immunocal on the synthesis of glutathione by peripheral blood mononuclear cells in vitro.

Glutathione determination. PBMC and tumor cells have a different base line GSH content; untreated PBMC have a GSH content of 4 nmoles/10^7 cells while MATB have 28 nmoles/10^7 cells and Jurkat T cells have 32 nmoles/10^7 cells. These are probably related to the different proliferative activity of tumor cells.

In the PBMC model when cells are cultures at varying concentration of IMMUNOCAL™, GSH increased in parallel with cell proliferation with a peak of GSH corresponding to an IMMUNOCAL™ concentration of 100 µg/ ml. At higher concentration (500 µg/ml) a drop of

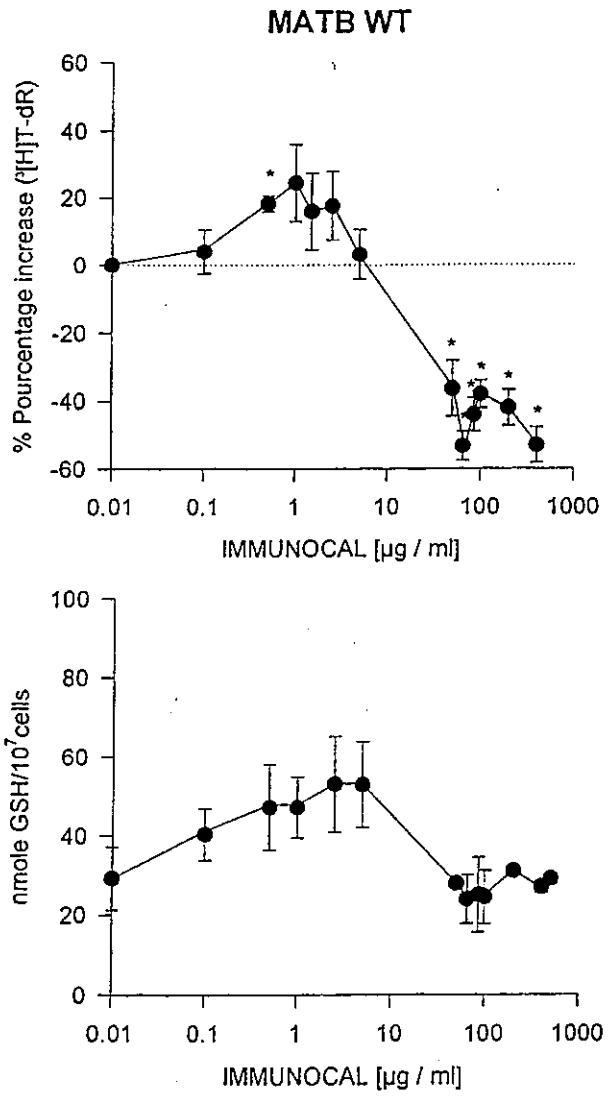

Figure 2. *Upper: Effect of Immunocal on the proliferation of MATB WT tumour cells in vitro.*
Lower: Effect of Immunocal on the synthesis of glutathione by MATB WT cells in vitro.

intracellular GSH was found which correlated with a drop of cellular proliferation from the levels obtained at 100 µg/ml (Figure 1).

In the cancer cell model either MATB or Jurkat T cells the inhibitory effect of IMMUNOCAL™ appears at much lower concentration such 5 to 50 µg/ml (Figure 2, 3), and this inhibition of proliferation clearly correlated with a drop in intracellular GSH (Figure 2).

In conclusion normal cells respond differently from cancer cells to the humanized whey protein concentrate IMMUNOCAL™ and this selective response in term of proliferation is correlated with the GSH intracellular metabolism of each type of cellular system.

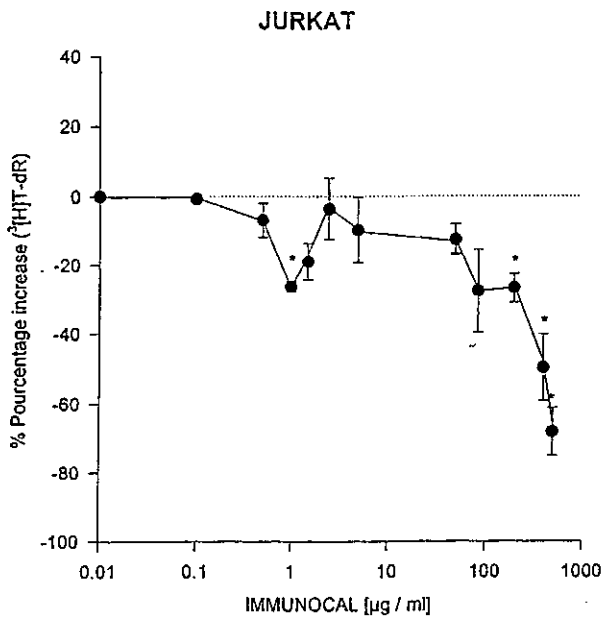

Figure 3. *Effect of Immunocal in the proliferation of JURKAT tumour cell in vitro.*

Discussion

This report demonstrates that it is possible to differentially manipulate GSH *in vitro* with this humanized Whey Protein Concentrate, depending whether normal lymphocytes or tumors cells are used. As demonstrated in our experiment normal lymphocytes cultured for three days with concentration of WPC at 100 µg/ml show an increase in intracellular GSH content from 4 nmoles/ 10^7 to 10 nmoles /10^7 cells, this increase in GSH correlates with an in increase in cell proliferation. On the contrary, the same experiment with the same concentration cystine delivery system of HWPC but in the tumors cell MATB and less in the Jurkat T cells showed a paradoxical drop in intracellular GSH and an inhibition in cellular proliferation. Viability tests confirm that this effect is not related to cytotoxic effect of HWPC.

Indeed previous *in vitro* studies have confirmed a direct inhibitory effect of whey protein on human cancer cells replication (10). In other human mammary cancer cell studies the inhibitory effect was found to be related to the serum albumin component of whey (11) and most recently to alpha lactalbumin another major component of whey protein concentrate (12). Feeding lactoferrin to mice inhibited the growth of solid tumors and in addition reduce lung colonization by melanomas (13). Serum albumin was found to exhibit, unlike other proteins a strong antimutagenic effect in an *in vitro* assay using hamster cells (14). Interestingly alpha lactalbumin, serum albumin and lactoferrin are extremely rich in cystine residue and the latter two proteins are also rich in glutamyl-cystine dipeptides necessary for GSH synthesis (15).

Recently an Australian group confirmed in rats the earlier observations of a Canadian research group in mice (16) demonstrating the incidence of intestinal tumor following dimethylhydrazine is less in animals fed WPC and casein than in those fed meat or soy bean diet, the whey protein fed animals showed the lowest tumour incidence and burden (17).

We confirm the inhibitory effect of humanized whey protein on tumor cell growth and propose as an explanation that WPC selectively inhibit GSH metabolism by using to its own detriment the mechanism of regulation of GSH metabolism of tumor cells.

Selective GSH modulation is an attractive concept in order to reduce radio- and chemotherapy toxicity. Humanized Whey Protein Concentrate is an effective cystine delivery system and seems to result in a selective *in vitro* and *in vivo* GSH modulation (15,17).

Work done in the late 1980's has demonstrated that an increase in tissue glutathione levels can be obtained by providing a diet containing adequate levels of whey protein (15). Whey protein, a constituent of milk, is made of serum albumin, lactoferrin, beta lactoglobulin, alpha lactalbumin, immunoglobulin. The glutathione modulating activity of dietary whey protein is dependent on cystine present in bovine serum albumin, alpha lactalbumin, lactoferrin and the glutamyl-cystine groups and homoglutathione (16). Administration of whey protein obviates the toxic effect of other known agents for increasing the intracellular level of GSH such as N acetyl cysteine.

In serum albumin there are 17 cystine residues per 66,000 MW molecule in lactoferrin 17 per 77,000MW molecule and in alpha lactalbumin 4 per 14,000MW molecule Whereas free cysteine does not represent an ideal delivery system because of it is toxic and it is rapidly metabolized, cystine in peptide released during digestion in the gastrointestinal tract is more stable than free amino acid More specifically, Meister has demonstrated that the gamma glutamyl-cystine precursors of GSH can easily enter into the cell and then be synthesized into GSH.

The selectivity that we demonstrated in our experiment may be explained by the fact that GSH synthesis is negatively inhibited by its own synthesis, and since as mentioned, baseline intracellular GSH in tumor cells is much higher than in normal cell it is easier to reach the level of a negative feed back inhibition in this cellular system than in a non tumor cellular system.

We recently reported in vivo selective modulation of GSH using the GSH prodrugs 2- L-Oxothiazolidine in animals bearing mammary carcinoma (18) These results substantiate the hypothesis formulated in this article since we demonstrated that the cysteine prodrug OTZ can enhance GSH in normal tissue while depleting GSH in tumor tissue.

In a recent phase 1-2 study using IMMUNOCAL™ in patients with metastatic carcinomas we demonstrated that in addition to a beneficial clinical effect, IMMUNOCAL™ had the ability to induce a sustained drop of GSH level toward normal in patients that presented progressive disease associated with a high intracellular GSH level. on the contrary patients who progressed while on study showed a trend toward higher blood lymphocyte GSH levels (19).

Assuming that the levels of GSH in lymphocytes reflect the amount of GSH found at the tumor levels (20). the ability of IMMUNOCAL™ to modulate selectively tumor GSH and systemic GSH level open a new area of nutriceutical manipulation of GSH in cancer patients treated with chemotherapy.

These *in vitro* data and the result of a preliminary clinical study indicates that this newly discovered property of humanized whey protein IMMUNOCAL™ may be a promising adjunct in the nutritional management of cancer patients about to undergo chemotherapy. Selective depletion of tumour GSH may in fact render cells more vulnerable to the action of chemotherapy and eventually protect normal tissue against the deleterious effect of chemotherapy.

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