Oxidative Stress in Cancer, AIDS, and Neurodegenerative Diseases

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Nutriceutical Modulation of Glutathione with a Humanized Native Milk Serum Protein Isolate, IMMUNOCAL™: Application in AIDS and Cancer

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NUTRITIONAL IMMUNOMODULATION AND ITS RELATION TO GLUTATHIONE SYNTHESIS

Fresh, raw milk includes the group of proteins that remain soluble in "milk serum." These proteins can be preserved in their native form if extracted carefully from their natural source.

In 1981 it was discovered that normal mice fed a milk serum protein concentrate (specially prepared under mild nondenaturing conditions) exhibited a marked increase in the humoral immune response to a T helper cell-dependent antigen (1). In the following years, numerous experiments confirmed the consistency of this phenomenon (2–10). Over a period of 12 years and based on these findings a humanized native milk serum protein isolate (HNMPI) named IMMUNOCAL™ was developed (Immunotec Research Corporation Ltd., Montreal, Quebec, Canada).

This property was found to be related, at least in part to a greater production of splenic glutathione (L-α-glutamylcysteinylglycine) (GSH) during the oxygen-requiring antigen-driven clonal expansion of the lymphocyte pool in animals fed with this bioactive HNMPI (9). Adequate levels of GSH are necessary for lymphocyte proliferation in the development of the immune response (11,12). Moderate but sustained elevation of cellular GSH was also found in the liver and the heart of healthy,

old mice fed with this HNMPI for a prolonged period. In addition, HNMPI markedly increased their life expectancy in comparison to control animals fed nutritionally equivalent diets (13).

Glutathione is of major significance in cellular antioxidant activity in what Meister called the "GSH antioxidant system" because it participates directly in the destruction of reactive oxygen compounds and also because it maintains in reduced form ascorbate (vitamin C) and α-tocopherol (vitamin E), which also exerts an antioxidant effect (14).

FUNCTION OF HNMPI AS A CYSTEINE DELIVERY SYSTEM

What ingredient in IMMUNOCAL™ makes it an effective "cysteine delivery system"?

Systemic availability of oral GSH is negligible in man (15) and there is no evidence for transport of GSH into cells (16). Thus, it has to be synthesized intracellularly. This occurs in two steps: (a) glutamylcysteine synthesis; (b) glutathione synthesis. Even though the inflow of cysteine, glutamate, and glycine might prove somewhat limiting under selected circumstances, numerous observations have shown that it is the transport of cysteine (or cystine, which usually is promptly reduced to cysteine on cell entry) which tends to be the rate-limiting event in GSH synthesis. whereas free cysteine does not represent an ideal delivery system (17) because it is toxic and is spontaneously oxidized. Cysteine present as the disulfide cystine released during digestion in the gastrointestinal tract is more stable than free amino acid. GSH synthesis is submitted to negative feedback inhibition by the end-product GSH. The disulfide bond is pepsin- and trypsin-resistant, but may be split by heat and mechanical stress (9). Cystine accounts for about 90% of the low-molecular-mass cysteine in the blood plasma, while reduced cysteine is present only at extremely low concentration (18).

In a comparative study, we found that commercial milk serum concentrates exhibiting far less bioactivity, including less GSH promoting activity, contain about half the amount of serum albumin (9) and 4 times less lactoferrin than HNMPI, expressed as percentage of total milk serum protein. IMMUNOCAL™ is produced in a proprietary lenient process which results in the preservation of the most thermolabile proteins in their native conformation.

In the serum albumin, there are 17 cystine residues per 66 kDa molecule and 6 Glu-Cys dipeptides (19); in lactoferrin there are 17 per 77 kDa molecule and 4 Glu-Cys dipeptides (20); and in the α-lactalbumin there are 4 cystines in a 14,000 kDa molecule

Table 1.

	Molecular Mass (kDa)	Residues	Cysteine residues per molecule	Cysteine $(Cys)_2$ (disulfide)	Glu-$(Cys)_2$
β-Lactoglobulin	18,400	162	5	2	0
α-Lactabumin	14,200	125	8	4	0
Serum albumin	66,000	582	35	17	6
Lactoferrin	77,000	708	40	17	4

Source: Refs. 19, 20.

(19). On the other hand, β lactoglobulin has only 2 cystines in a 18,400 kDa molecule (19), and IgG1, the predominant immunoglobulin in cow whey, has only 4 disulfide bridges in a 166,000 kDa molecule (Table 1). In addition, Meister and colleagues (16) have demonstrated that the γ-glutamylcysteine (Glu-Cys) precursors of GSH can easily enter the cell and there be synthesized into GSH. It thus become noteworthy that the most labile milk proteins–, serum albumin and lactoferrin–are those which contain these putative GSH-promoting peptide components.

Finally, the bioavailibility of the presumed active component (cystine and Glu-Cys group) may be influenced by the coexistence of the other proteins throughout the digestive–absorptive process.

This newly discovered property of HNMPI was found to be independent of its nutritional value, as other proteins of similar nutritional efficiency do not exhibit this unique property (1–10). The concept that a specific biological activity can exist in addition to and independent of the systemic effect of IMMUNOCAL™ as a good protein source is further substantiated by recent in vitro assays (21).

The dietary provision of cystine is particularly relevant to the immune system. The coordinated response of macrophages and lymphocytes in the T cell-mediated immune response is regulated, in part, by macrophage cystine uptake and subsequent release of reduced cysteine into the local environment for uptake by lymphocytes. When the antigen-presenting macrophages come into close contact with antigen-specific T cells, they supply these cells with additional amounts of cysteine and thereby raise their intracellular GSH level (18).

The validity of this assumption is confirmed by the demonstration that the immunoenhancing and GSH-promoting (data not shown) effect of IMMUNOCAL™ is abolished by buthionine sulfoximine, which inhibits γ-glutamylcysteine synthetase, the initial step in GSH synthesis (17).

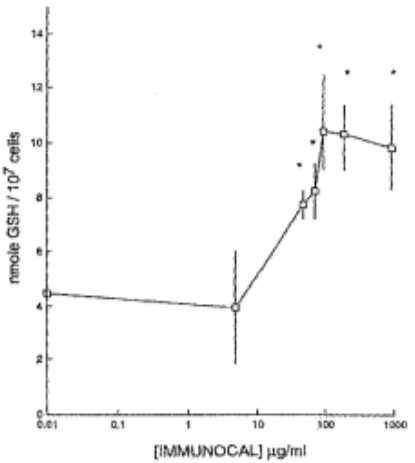

Figure 1. Incubation of PBMC for 72 h in the presence of various amounts of IMMUNOCAL™. Each point represents the mean ±SD of 3 measurements of intracellular glutathione. *$p < 0.05$.

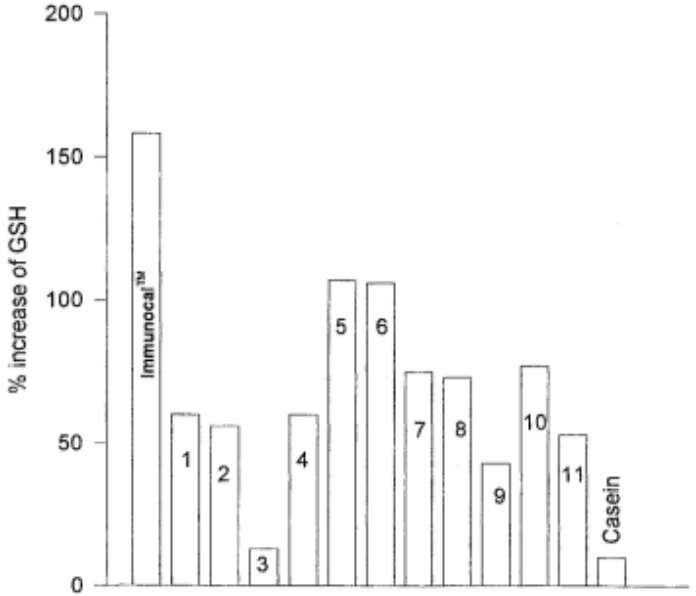

Figure 2 Incubation of PBMCs for 72 h in the presence of IMMUNOCAL™ and other serum milk products: Percentage increase in glutathione.

IN VITRO MODULATION OF INTRACELLULAR GLUTATHIONE BY IMMUNOCAL™

We demonstrated that normal human lymphocytes cultured for 3 days with HNMPI 100 μg/ml show an increase in intracellular GSH content from 4.5 ± 0.4 to 10.5 ± 3.4 nmol/10^6 cells, p <0.01 (Figure 1). This increase in GSH correlates with an increase in cellular proliferation measured by thymidine incorporation (data not shown). The

Table 2 Presence of Cytopathic Effects in MT-4 Cells

	$TCID_{50}$/well[a]			
IMMUNOCAL™ (μg/ml)	2000	200	20	2
0	+++	++	+	–
1	+++	++	+	–
10	++	+	+	–
100	–	–	–	–
500	–	–	–	–
1000	–	–	–	–

[a]+ Presence of cytopathic effects; – absence of cytopathic effects.

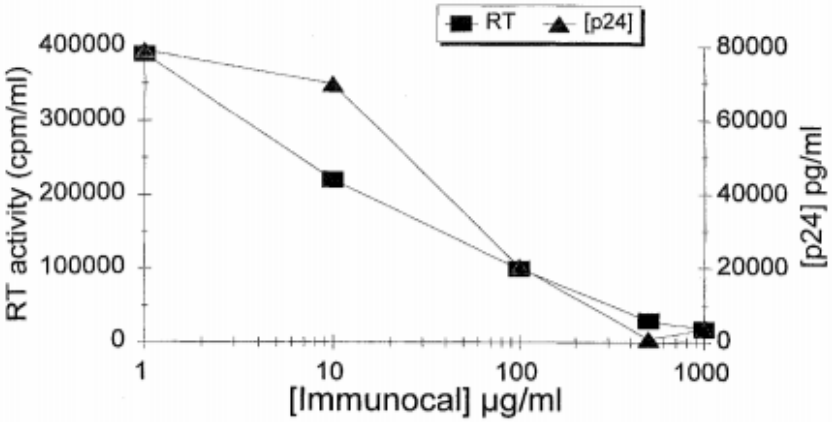

Figure 3. IMMUNOCAL™ has been shown to inhibit HIV replication.

increase in GSH is dose-dependent and has not been found for casein or for any commercially available milk serum protein concentrate (Figure 2).

IN VITRO ANTI-HIV and ANTIAPOPTOTIC ACTIVITY OF HNMPI

Clinically, there is direct evidence that HIV infection is associated with a GSH deficiency in the peripheral blood mononuclear cells (PBMC) (18). The depletion of intracellular GSH suggests an association between oxidative stress and HIV infection. Oxidative stress may be one of the mechanisms that contribute to disease progression and the wasting syndrome through mediators of inflammation such as TNF-α and IL-6. During this period of progression, glutathione is consumed owing to an increase in oxidative stress. GSH

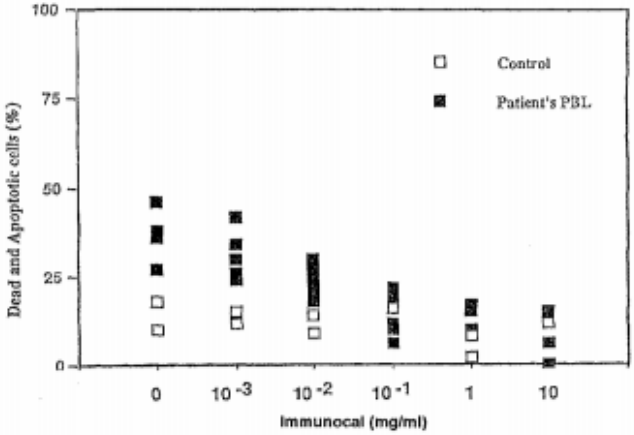

Figure 4. Inhibition of early cell death by IMMUNOCAL™.

depletion, a consequence of chronic oxidative stress, is part of the spectrum of HIV infection. GSH has, in addition, a crucial role in lymphocyte function and cell survival.

IMMUNOCAL™ functioning as a cysteine delivery system can enhance GSH synthesis in vitro (Figure 1) and inhibits HIV replication on a cord mononuclear cell system infected by HTL V-IIIB (Figure 3). IMMUNOCAL™ also inhibits the formation of syncitium between infected and noninfected cells. The inhibition of syncitium formation occurred at the same concentration as inhibition of HIV replication (Table 2). This viral inhibition was not associated with any cytotoxicity. IMMUNOCAL™, via its GSH-promoting activity, reduces apoptosis in HIV-infected cells. Apoptosis was evaluated by flow cytometry on PBMC from HIV-infected individuals (Dr. R. Olivier, AIDS and Retrovirus Department, Pasteur Institute). HIV-infected PBMC cultured at concentrations of IMMUNOCAL™ of 100 μg/ml or higher were less prone to die of apoptosis than untreated cells: 15% ± 2.6% vs. 37% ± 2.4, $p<0.001$ (Figure 4).

HNMPI SUPPLEMENTATION IN AIDS AND WASTING SYNDROME

Based on these preclinical data, we conducted a Canadian clinical trial (Canadian HIV Trials Network) with IMMUNOCAL™ in children with AIDS and wasting syndrome. The major objective was to evaluate the effect of oral supplementation with IMMUNOCAL™ on nutritional parameters and intracellular blood lymphocyte GSH concentration in children with AIDS and wasting syndrome. This was an open single-arm pilot study of 6 months duration. Wasting syndrome and severe weight loss within the 6 months preceding entry into the study was an absolute criterion for entry.

IMMUNOCAL™ was administered twice a day as a powder diluted in water. In some patients, IMMUNOCAL™ was administered via nasogastric tube when necessary. The administered starting dose was based on 20% of the total daily protein requirement and was increased by 5% each month over 4 months to reach 35% of the total protein intake at the end of the study. The total duration of the study was 6 months.

Weight, height, triceps skinfold and mid-arm muscle circumferences, CD4/CD8 counts, and peripheral lymphocyte GSH concentrations (measured by spectrophotometric assay) were measured monthly. Energy intake was assessed by the use of two independent 2-day food records with a 2–3 week period between the food records. Each food record included a weekday and a weekend, and the average of these records was calculated to reflect the daily nutritional intake. Out of 14 patients enrolled, 10 were evaluable. The ages of the patient were from 8 months to 15 years. The 10 patients studied were enrolled in four different centers across Canada: Montreal Children's Hospital (Dr. S. Baruchel), The Hospital For Sick Children Toronto (Dr. S. King), Children's Hospital for Eastern Ontario (Dr. U. Allen), and Centre Hospitalier Laval Quebec (Dr. F. Boucher). Of the 4 remaining patients, 2 lacked compliance after 2 months while the other 2 died of AIDS progressive disease within the first 2 months of entry into the study. None of the deaths was related to the tested product.

None of the patients experienced any major toxicity such as diarrhea or vomiting or manifestation of milk intolerance. One patient had to stop IMMUNOCAL™ transiently for minor digestive intolerance such as nausea and vomiting (< twice/day) at month 3 and was subsequently able to restart the treatment without any problem.

At the end of the study, all patients experienced a weight gain in the range of 3.2% to 22% from their starting weight. The mean weight gain for the group was 8.4% ± 5.7%. On analysis of the mean percentage of requirement nutrient intake (RNI) per month for all

Table 3 Changes from Baseline (expressed as percentage) at Weeks 24 and 36 in Weight, Anthropometric Measurements and GSH in Patients Treated with IMMUNOCAL™

	Weight change (%)		Mid-arm muscle circumference change		Triceps skinfold change (%)		PBMC GSH change (%)	
Patient no.	wk 24	wk 32	wk 24	wk 32	wk 24	wk 32	wk 24	wk 32
1	22.1	29.8	9.5	14.3	50.0	25.0	12.2	–9.0
2	14.0	17.3	18.7	25.3	20.0	–20.0	84.0	56.0
3	5.1	9.2	–3.0	–2.0	–17.0	–3.0	37.0	55.0
4	3.8	3.4	4.2	NA	–42.0	NA	305.0	550.0
5	7.1	4.5	13.1	11.4	–24.0	–16.0	–18.0	14.3
6	3.7	5.6	–2.0	–2.0	16.0	16.0	7.1	174.0
7	2.5	NA	5.0	NA	–13.0	NA	54.2	NA
8	14.2	18.2	–3.1	2.0	41.0	43.0	17.3	62.4
9	8.9	7.9	–4.0	–8.0	–30.0	–39.0	–6.6	50.9
10	7.0	NA	1.0	NA	41.0	NA	–1.6	NA

NA

the patients, no correlation was found between the weight gain and any significant increase in the mean percentage of RNI, suggesting reduced catabolism rather than an anabolic effect of IMMUNOCAL™. Six of ten patients have demonstrated an improvement in their anthropometric parameters such as triceps skinfold or mid-arm muscle circumference independently of an increase in energy intake (Table 3).

Two groups of patients were identified in terms of GSH modulation: responders and nonresponders. The responders were those who started the study with a low GSH level.

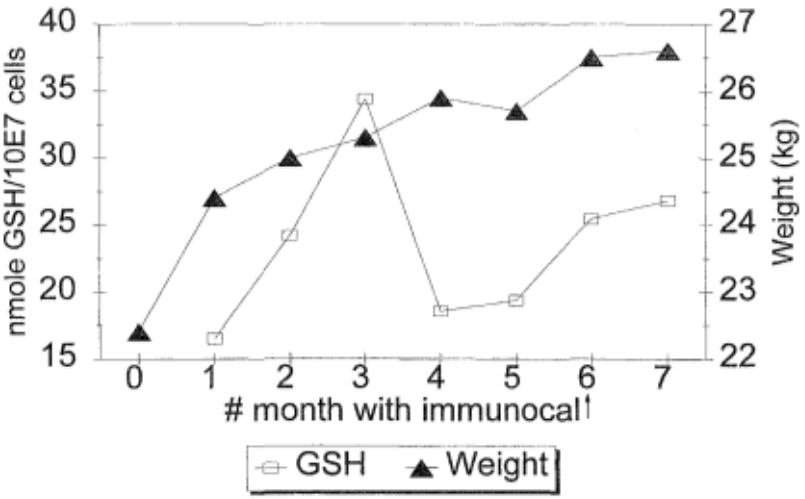

Figure 5. Intracellular glutathione in. HSC 4. Each point represents the mean ±SD of 3 measurements. ↑ indicates end of study.

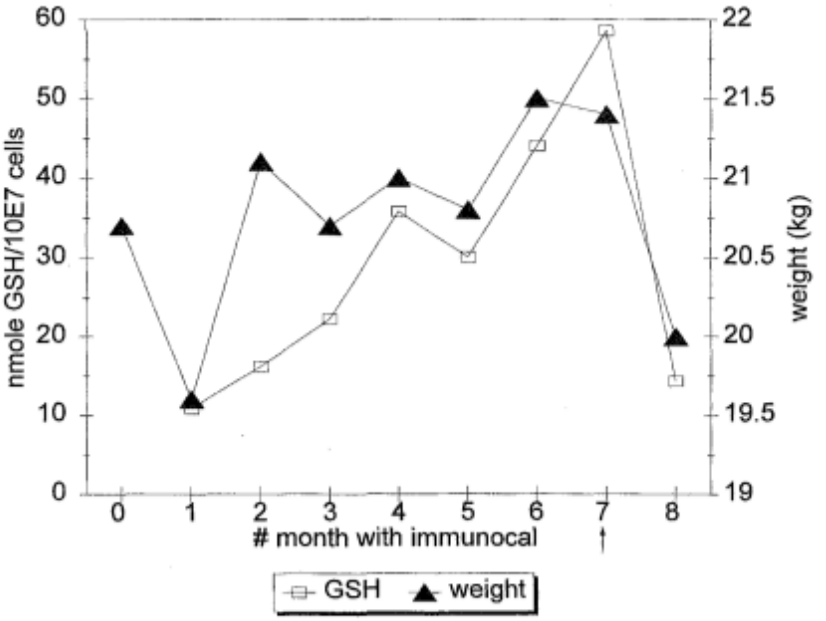

Figure 6. Intracellular glutathione in CHUL 1. Each point represents the mean ±SD of three measurements. ↑ indicates end of study.

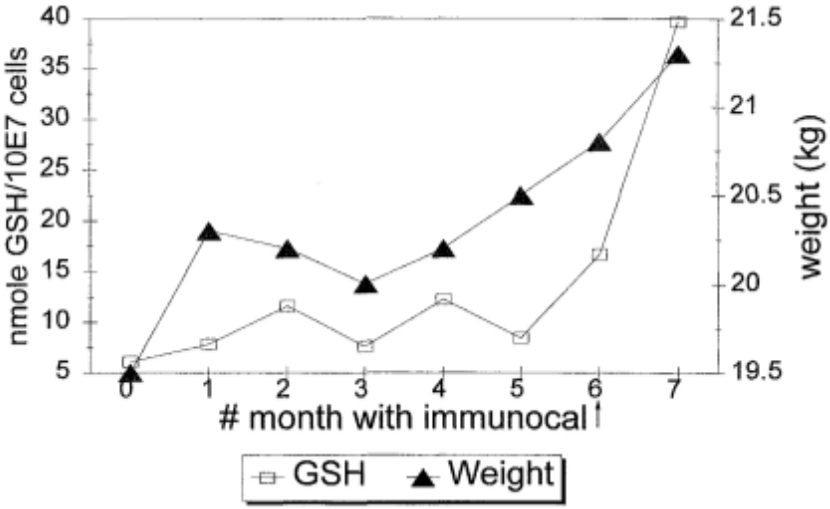

Figure 7. Intracellular glutathione in MCH 3. Each point represents the mean ±SD of three measurements. ↑ indicates end of study.

The nonresponders were those who started with a normal GSH level. A positive correlation was found between increase in weight and increase in GSH (Figures 5,6,7). No changes were found in terms of blood lymphocyte CD4 cell count, but 2 patients exhibited an increase in the percentage of their CD8 cells and 4 patients showed a trend toward an increase in the number of NK cells.

In conclusion, this pilot study demonstrates that IMMUNOCAL™ is very well tolerated in children with AIDS and wasting syndrome and is associated with an amelioration of the nutritional status of the patient as reflected by weight and antrhopometric parameters. Moreover, the GSH-promoting activity of IMMUNOCAL™ in vivo seem to be validated in 6 out of 10 patients. An international multicenter double-blind randomized study is currently under way in France and Canada in adults patients with AIDS and wasting syndrome.

SELECTIVE GLUTATHIONE MODULATION OF BREAST CANCER CELLS AND IMPACT ON CANCER CELL GROWTH

The specific involvement of GSH in the carcinogenic process is supported by the major role played by this compound in the detoxification of carcinogens by conjugation (26). We demonstrated that feeding GSH-promoting HNMPI to mice chronically treated with dimethylhydrazine (DMH) significantly reduces the number and size of colon carcinomas induced by DMH (27,28). These colon tumors appear to be similar to those found in the human insofar as the type of lesions and the chemotherapeutic response characteristics are concerned (26). HNMPI feeding appears to exert an inhibitory effect not only on the initiation (27) of cancer, but also on the progression of tumors (28).

Recently, a direct inhibitory effect of HNMPI in human cancer cell replication was confirmed (21,29,30). In other human cancer cell studies, the inhibitory effect was found to be related to the serum albumin component of milk serum (31) and most recently to α-lactalbumin (32). Feeding lactoferrin to mice inhibited the growth of solid tumors and in addition reduced lung colonization by melanomas (33). Unlike other proteins, serum albumin was found to exhibit a strong antimutagenic effect in an in vitro assay using hamster cells (34). It is therefore noteworthy that in this HNMPI we have succeeded in concentrating serum albumin, α-lactalbumin, and lactoferrin, all containing a significant number of GSH precursors. A possible explanation for these newly discovered properties of dietary milk serum protein may be found in recent findings on the role of GSH in tumor biology (35).

The search for ways to inhibit cancer cells without injuring normal cells has been based over the years on a vain effort to identify the metabolic parameters in which cancer cells are at variance with normal cells. One such function could well be the all-important synthesis of cellular GSH.

Recent experimental evidence has revealed an intriguing response of tumor versus normal cells to GSH synthesis-promoting compounds. Cellular GSH levels have been found to be several times higher in human cancer cells than in adjacent normal cells (35). This finding is presumably related to their proliferative activity. In fact, cancer is the only condition in which elevation of such a tightly regulated system as GSH has been reported. However, when a cysteine- and GSH-promoting compound such as 2-L-oxothiazolidine-4-carboxylate (OTZ) was added to cultured human lung cancer cells exhibiting very high levels of GSH at the outset, no intracellular increase was noted, whereas GSH increased substantially in normal cells (35). This differential response is even more pronounced in vivo. We demonstrated that in tumor-bearing rats, OTZ treatment was actually found to deplete GSH in the tumors (36).

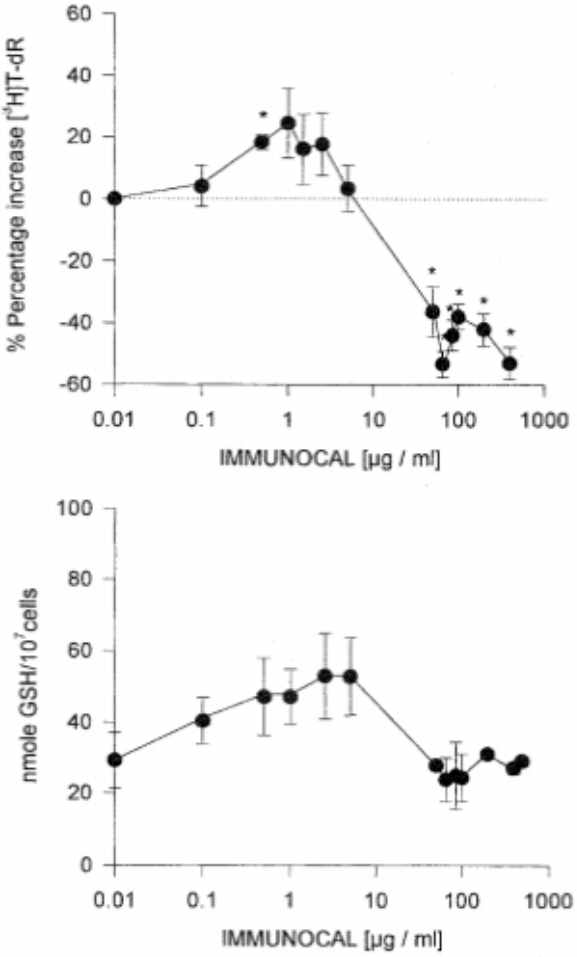

Figure 8. Intracellular glutathione in MATB WT. Each point represents the mean ±SE of three measurements. $^*p < 0.05$.

More specifically, an in vitro assay showed that, at concentrations that induce GSH synthesis and proliferation in normal human cells (Figure 1), IMMUNOCAL™ caused GSH depletion and inhibition of proliferation of cells in a rat mammary carcinoma (Figure 8) and Jurkat T cells (Figure 9) (21).

The selectivity demonstrated in these experiments 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 cells, it is easier to reach the level at which negative feedback inhibition occurs in this cellular system than in a nontumor cellular system.

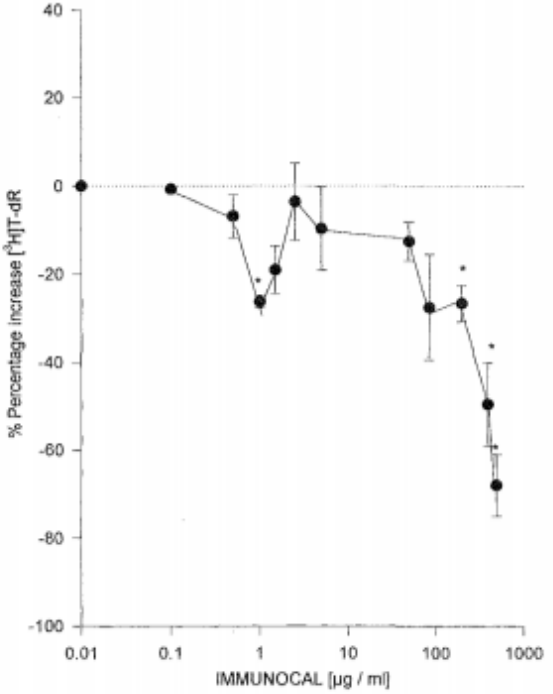

Figure 9. Intracellular glutathione in JURKAT. Each point represents the mean ±SE of three measurements.

HNMPI IN CANCER CLINICAL TRIALS

On the basis of these experiments, 5 patients with metastatic carcinoma of the breast, 1 of the pancreas, and 1 of the liver were fed 30 g of IMMUNOCAL™ daily for 6 months. In 6 patients, the blood lymphocyte GSH levels were substantially above normal at the outset, probably reflecting high tumor GSH levels. At completion of the 6 months of daily supplementation, 2 patients exhibited signs of tumor regression, normalization of hemoglobin and peripheral lymphocytes counts, and a sustained drop of lymphocyte GSH levels toward normal. Two patients showed stabilization of the tumor and increases in hemoglobin levels. In 3 patients, the disease progressed with a trend toward higher lymphocytes GSH levels (37).

A major problem in the use of chemotherapeutic agents in cancer therapy is the protection offered by the defense mechanisms of cancer cells. An important element of protection is represented by GSH, which is an effective detoxification agent that is relatively abundant in tumor cells. Indeed, when GSH synthesis is inhibited by buthionine sulfoximine (BSO), the activity of several chemotherapeutic agents such as alkylating agents is increased and drug resistance can be reversed (36–38). However, the concomitant depletion of GSH in normal cells greatly limits the practical usefulness of this modality of treatment.

We recently demonstrated that a selective GSH prodrug such as OTZ protects some normal tissue (36) but also potentiates the activity of some alkylating agents (38). The apparently selective depletion of tumor GSH levels by provision of a natural precursor of GSH as contained in IMMUNOCAL™ seems to be associated with inhibition of proliferation of cancer cells in vitro. This natural precursor of GSH favorably influences the GSH synthesis in normal cells. These in vitro and preliminary clinical results indicate that this newly discovered property of HNMPI may be a promising adjunct to the nutritional management of cancer patients undergoing chemotherapy. We are currently developing a phase II study in breast carcinoma, attempting to confirm that this selective depletion of GSH may, in fact, render tumor cells more vulnerable to chemotherapy and eventually protect normal tissue against the deleterious effect of chemotherapy.

ANALOGY BETWEEN HNMPI IMMUNOCAL™ AND HUMAN MILK

Human milk contain about 80% of whey protein and 20% of casein. The opposite is true for cow milk. An analysis of the mass ratio of casein to whey protein in milk from various mammals clearly indicates that human milk has the lowest ratio in any mammalian species (39). On the basis of our laboratory studies showing the immunoprotective and anticancer effects of cow whey protein concentrate, it is tempting to speculate that this predominance of whey proteins in human milk is advantageous and thus represent an evolutionary adaptation.

Scientific data based on the similarity between the bioactive components of this native milk protein isolate (HNMPI) of cow milk, IMMUNOCAL, and human whey protein appear to substantiate this theory, as will now be discussed in more detail.

It is well known that breast feeding is superior to the use of cow milk-based formulas of similar nutritional efficiency for the health of human babies. Breast feeding protect against otitis media, and pneumonia (40,41). Mothers milk also has a protective effect on the incidence of several types of childhood cancer including leukemia, lymphomas, bone tumors, and brain tumors (42). Children who are artificially fed or are breast fed for only a short period of time are more at risk for developing several types of cancer before the age of 15 years as compared to long-term breast feeders (43). Thus, the concept of a biological activity in addition to but independent of the nutritional efficiency, formulated to describe the immunoenhancing and GSH-promoting activity of the HNMPI IMMUNOCAL™, may indeed apply to the breast feeding of neonates and infants. Glutathione synthesis appears to be the crucial factor in the health benefit of HNMPI.

It may then be appropriate to identify the features common to HNMPI and human whey proteins that are capable of influencing GSH synthesis in the host. Cysteine, a crucial limiting factor in the synthesis of GSH, is about as abundant in cow's whey protein as it is in whole human milk proteins and several times more abundant than in cow's whole milk (39), since most caseins contain either no cysteine or one or two cysteine residues (19). As mentioned earlier, our studies showed that the most thermolabile milk proteins, namely, serum albumin, α-lactalbumin, and lactoferrin, are crucial to expression of the bioactivity of HNMPI. As shown in Table 1, these proteins are rich in cystine and glutamylcystine residues, natural precursors of GSH. The presence of these dipeptides in the product IMMUNOCAL™ is a characteristic shared with human milk (Table 4).

Traditionally, it has been advocated that "humanized" cow milk should contain more α-lactalbumin because this protein is twice as abundant in human milk. On the basis of our experimental findings, we propose instead that the principal health factor in human milk,

Table 4. Protein Composition of Cow and Human Milk
Composition (g/litre)

Component	Cow milk	Human milk	
Casein (g/L)	26	3.2	(0 or 2 cysteine/molecule no disulfide bond)
β-Lactoglobulin (g/L)	3.2	Negligible	
α-Lactalbumin (g/L)	1.2	2.8	
Serum albumin (g/L)	0.4	0.6	
Lactoferrin (g/L)	0.14	2.0	
Total cystine (mol/L)	8.19×10^{-4}	13.87×10^{-4}	
Total cystine (mg/g protein)	6.4	38.7	

Source: Ref. 19; Jennes R. Inter-species comparison of milk proteins. In Fox, ed. Developments in dairy chemistry–1. New York: ASP; 1982:8

not denaturated by heat pasteurization, is due to the predominance of the thermolabile proteins rich in cystine and containing the Glu-Cys dipeptide which are characteristic of the bioactive HNMPI, namely, serum albumin, α-lactalbumin, and lactoferrin. This HNMPI differs from other commercially available milk serum protein concentrates in having a relatively high content of serum albumin (about 10%), lactoferrin (about 0.65%), and α-lactalbumin (about 28%). The variety of diseases against which breast feeding appears to be effective suggest a broader protective mechanism involving cellular GSH and its effect on free radicals, lymphocyte proliferation, and detoxification of carcinogens and other xenobiotics.

CONCLUSION

The biological activity of the proteins isolated from cow's milk in IMMUNOCAL™ depends on the preservation of those labile proteins which share with the predominant human milk proteins the same extremely rare GSH-promoting components. Cellular GSH depletion has been implicated in the pathogenesis of a number of degenerative conditions and disease states including Parkinson's, Alzheimer's, arteriosclerosis, cataracts, cystic fibrosis, malnutrition, aging, AIDS, and cancer (9).

This newly discovered nutriceutical modulation of GSH by the use of humanized native milk serum protein isolate of bovine origin in AIDS and cancer may well find other applications in disease where oxidative stress and pathology of GSH metabolism are largely implicated. Extensive pharmacoepidemiological study of GSH metabolism and standardized methods of measurement of intracellular GSH applicable in clinical trials are needed in order to better define the clinical application of this new type of therapy.

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