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ORIGINAL CONTRIBUTION

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Effects of long-term supplementation with whey proteins on plasma glutathione levels of HIV-infected patients

Summary *Background* HIV infection is characterized by an enhanced oxidant burden and a systemic deficiency of the tripeptide glutathione (GSH), a major antioxidant. The semi-essential amino acid cysteine is the main source of the free sulfhydryl group of GSH and limits its synthesis. Whey proteins are rich in cysteine as well as in GSH precursor peptides. *Aim of the study* In order to evaluate the effects of whey supplementation on plasma GSH levels, HIV-infected patients were treated with whey proteins for a period of six months. *Methods* In a double blind clinical trial, 30 patients were randomized to a daily dose of 45 g whey proteins of either Protectamin® (Fresenius Kabi, Germany) or Immunocal® (Immunotec, Europe) for 2 weeks. Eighteen patients (16 male, 42 ± 9.4 yr, 249 ± 99 CD4+ lymphocytes/l) continued the trial with a daily dose of 45 g of Protectamin for six months. *Results* Pretherapy, total plasma GSH levels (Protectamin: 1.92 ± 0.6 μM; Immunocal: 1.99 ± 0.9 μM) were less than normal (2.64 ± 0.7 μM, p = 0.03). After two weeks of whey protein supplementation, plasma total GSH levels increased in the Protectamin group by 44 ± 56 % (2.79 ± 1.1 μM, p = 0.004), while the difference in the Immunocal group did not reach significance (+24.5 ± 59 %, 2.51 ± 1.48 μM, p = 0.43). Consequently, all patients continuing the trial were openly switched to Protectamin. After six months, total GSH plasma levels were still significantly elevated compared to baseline (day 1: 1.95 ± 0.8 μM vs. month 1: 2.18 ± 0.8 μM, p = 0.19; month 3: 2.39 ± 0.9 μM, p = 0.056; month 6: 2.47 ± 0.8 μM, p = 0.033). Body weight, T-cell counts, and other clinical parameters did not change. The most common mild side effect was intestinal disturbance; severe adverse events did not occur. *Conclusion* Supplementation with whey proteins persistently increased plasma glutathione levels in patients with advanced HIV-infection. The treatment was well tolerated. A larger long-term trial is clearly warranted to evaluate whether this positive influence on the glutathione metabolism translates into a more favorable course of the disease.

Key words HIV – glutathione – cysteine – whey protein – oxidants – antioxidants

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Introduction

The tripeptide glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) is essential for metabolic and cell-cycle related functions in virtually all cells. Its ability to directly scavenge free radicals and to act as a co-substrate in the glutathione peroxidase catalyzed reduction of H_2O_2 and lipid hydroperoxides makes GSH central to defense mechanisms against intra- and extracellular oxidative stress [1]. In addition glutathione and glutathione transferases are major components among the mechanisms involved in the metabolism of xenobiotics [2, 3]. HIV infection is characterized by a systemic glutathione deficiency [4, 5] which develops only weeks following infection [5, 6], and by increased oxidative stress [7, 8]. Surprisingly, even given these facts, the role of the glutathione system in the pathogenesis of HIV-associated disease and its impact on the clinical course of the infection is still unclear [9, 10]. However, there is consen-

sus that HIV-infected individuals have subnormal concentrations of GSH and cysteine, as well as of glutamine, the second important glutathione precursor amino acid, in various body compartments [4, 11–14]. These findings suggested that therapeutic intervention with glutathione or glutathione precursors could stop or at least delay the normal progression of the disease [15, 16]. In a double blind, placebo controlled clinical trial low glutathione levels in CD4+ lymphocytes correlated strongly with survival in HIV-infected individuals. Oral administration of N-acetylcyteine, a glutathione precursor, restored intracellular GSH levels and significantly increased survival rates of patients in a two year open-label follow-up study [17]. These findings were confirmed and extended in a recent placebo controlled trial with oral NAC. Not only were deficient plasma GSH levels restored, but also immunological functions were enhanced [18]. As a consequence, strategies to increase plasma glutathione concentrations and to compensate for the oxidant-antioxidant imbalance were again brought into focus of clinical research and patients' interest.

Sufficient cysteine supply is essential for the maintenance of the glutathione pool [19]. Thus, highly purified cysteine-rich preparations of whey proteins have been proposed as ideal dietary supplements. A previous study including 3 HIV-infected patients who were orally treated with 40 g whey protein per day for three months revealed an increase of GSH levels and body weight [20]. With this as background the aim of this study was to evaluate in HIV-infected individuals 1) the influence of long-term (6 months) oral whey protein supplementation on plasma GSH concentrations, and 2) the practicability and safety of this form of treatment.

Methods

Study population

The study population included HIV-seropositive individuals aged > 18 years, with a diagnosis of HIV infection confirmed by ELISA and Western blot analysis, a CD4+ lymphocyte count < 300 /µl, and a CD4/CD8 ratio < 1.2. Patients had to be clinically stable, i. e., absence of severe infection, and life expectancy had to be > 6 months. Exclusion criteria were severe diarrhea, intolerance against milk products, drug addiction or relevant concomitant diseases. None should consume excessive amounts of products likely to alter GSH levels (e. g., N-acetylcysteine, alcohol, acetaminophen) during 3 months before the study. Thirty HIV-seropositive individuals completed the two week Immunocal vs. Protectamin phase of the trial [21]. Eighteen patients (16 male, 2 female, 42.5 ± 9.4 yr), 9 patients of the Immunocal arm and 9 patients of the Protectamin arm, among them 15 homosexuals, 1 former drug abusers, and 2 patients with a history of sexual contact with an infected partner, continued the six month open-label Protectamin extension phase of the trial (Tables 1 and 2). All patients were receiving antiretroviral therapy, consisting of 2 to 4 drugs. In 3 patients the antiretroviral therapy had been changed during the 6 month study period. For comparison, 10 healthy individuals (7 male, 3 female; mean age 31.2 ± 3.3 yr; weight 72.2 ± 9 kg) were evaluated. All were HIV-seronegative by ELISA.

Table 1 Study population (day 1) of the randomized 2-week phase

	Protectamin (n = 15)	Immunocal (n = 15)
Age (yr)	43.4±9.2	42.7±10.3
Sex (male/female)	11/4	14/1
Height (cm)	174.5±8.9	176.0±5.21
Weight (kg)	70.0±9.9	71.8±10.1
BMI (kg/m^2)	22.5±2.1	23.0±3.4
Smoker (no)	7	8
CDC classification		
C3	11	11
C2	0	1
B3	2	1
B2	2	2
Creatinine (mg/dl)	1.0±0.2	1.0±0.2
GOT (U/l)	33.6±15.4	30.6±13.5
GPT (U/l)	35.6±18.6	33.9±20.8
Plasma protein (g/dl)	80.0±0.4	8.0±0.5
CD4± lymphocytes (/µl)	241±95	226±122
CD4/CD8 ratio	0.41±0.37	0.30±0.2
Pretreatment GSH (µM)[a]	1.92±0.6	1.96±0.9
Posttreatment GSH (µM)[b]	2.79±1.2	2.51±1.48
No. of dropouts[c]	6	6

[a] Mean ± SD of total plasma GSH levels of all patients at day 1
[b] Mean ± SD of total plasma GSH levels of all patients at day 15
[c] Number of patients who refused to participate in the long-term trial

Table 2 Patients characteristics of the follow-up group

	(n = 18)
Age (yr)	42.5±8.7
Sex (male/female)	16/2
Height (cm)	175.0±7.3
Weight (kg)	69.1±9.0
BMI (kg/m^2)	22.5±2.1
Smoker (no)	12
CDC classification	
C3	16
C2	1
B3	1
Creatinine (mg/dl)	0.97±0.1
GOT (U/l)	29.8±13
GPT (U/l)	29.9±14
Plasma proteins (g/dl)	7.9±0.8
CD4± lymphocytes (/µl)	248.9±99.7
CD4/CD8 ratio	0.35±0.29

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Study design

Following a run-in period of one week to establish baseline levels for all study parameters, patients were randomized in a 1:1 ratio to receive 45 g whey protein per day of one of two different formulas (Protectamin®, Fresenius, Germany or Immunocal®, Immunotec, Europe). One patient dropped out due to gastrointestinal disturbance. Eighteen of the remaining 29 patients agreed to continue treatment with Protectamin in an open-label fashion for the total study period of six months. Eleven patients declined to take part in the long-term study period. Dropout rates and reasons for dropping out (private motives unrelated to the trial (n = 4), inconvenience of supplement preparation (n = 5), negative effect on appetite (n = 2)) were similar in both arms. After three months 2 patients, and after 6 months 3 more patients left the study prematurely. GSH levels, peripheral blood leukocytes including CD4+ and CD8+ lymphocytes, and clinical status including routine clinical parameters were evaluated at the beginning of the trial, and after one, three, and six months. Compliance of the patients during the trial was checked routinely at each visit by counting empty whey protein bags and by directly addressing the question of compliance. The protocol was approved by the Institutional Review Board of the University of Mainz and conducted in accordance with the Declaration of Helsinki. All subjects gave informed consent for the study.

Diet

Patients received either Protectamin or Immunocal (Table 3). The production process is distinct with a lower isolation temperature (< 72 °C) for Immunocal. Vanilla flavor was added in both formulas. The protein content ranges from 75–95 % with a fat content of 0–6 %. In both formulas the content of heavy metals was below the detection limit. No vitamins were added. The products were supposed to be taken in 3 equal portions of 15 g per day mixed with a nutritional adjuvant (e. g., milk, yogurt, buttermilk).

Biologic samples

Venous blood was obtained by standard techniques always in the morning after overnight fasting. Care was taken to avoid hemolysis, and blood samples were processed immediately.

Table 3 Amino acid and mineral content of the whey protein formulas. These data were kindly provided by Fresenius Kabi (Bad Homburg, Germany).

	Immunocal (g/100 g)	Protectamin (g/100 g)
Amino acid		
Aspartatic	9.80	10.50
Glutamic	16.37	17.48
Serine	3.56	5.58
Glycine	1.64	1.84
Histidine	2.04	1.73
Arginine	2.30	2.24
Threonine	4.63	6.97
Alanine	4.80	4.87
Proline	3.76	6.01
Tyrosine	3.76	1.97
Valine	4.53	6.06
Methionine	2.13	2.27
Isoleucine	6.41	6.32
Leucine	12.56	9.96
Phenylalanine	4.00	3.15
Lysine	10.68	9.19
Tryptophane	2.86	1.53
Cysteine/Cystine	4.17	2.28
Minerals		
Sodium	0.3	0.1
Potassium	0.5	0.5
Calcium	0.4	0.3
Chloride	0.4	0.1
Phosphate	0.3	0.3
Magnesium	0.3	0.1

Glutathione levels and form

Concentrations of total, reduced, and oxidized glutathione in plasma were quantified with minor modifications of standard methods, as previously described [4, 22]. In brief, to determine total glutathione levels (i. e., reduced glutathione, glutathione disulfide (GSSG)), plasma was mixed with an equal amount of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.1 M potassium phosphate, pH 7.5, containing 17.5 mM ethylenediaminetetraacetic acid (EDTA). The samples were centrifuged (2000 g, 10 min), and aliquots (50 µl) of the supernatants were added to cuvettes containing 0.5 U of GSSG reductase in 0.1 M potassium phosphate, pH 7.5, containing 5 mM EDTA. After incubation for 1 min at room temperature, the assay reaction was started by adding 220 nM of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in 0.1 M potassium phosphate, pH 7.5, containing 5 mM EDTA in a final volume of 1 ml. The rate of reduction of DTNB was recorded spectrophotometrically at a wavelength of 412 nm (Beckman DU–70 spectrophotometer). Determination of the total glutathione concentration was based on standard curves generated from known concentrations of GSSG (0.125 to 4 µM) in phosphate buffered saline, pH 7.4. All measurements were carried out in duplicate.

Routine laboratory parameters

White blood cells, cell differentials, and CD4+ and CD8+ lymphocytes were quantified with standard methods.

Statistical analysis

Statistical analysis was performed using REPORT 6.1 and Smart Test 1.3 software. Evaluation of efficacy was done by intention-to-treat analysis. The change from baseline plasma glutathione levels at month six was defined as the primary end point. Secondary end points were changes from baseline plasma levels of glutathione, and of CD4/CD8 ratios at months 1 and 3. Other secondary end points were amount and severity of infections, and side effects which were monitored throughout the whole study period. Unless otherwise specified, all data are given as mean ± standard deviation. The multivariate Wilcoxon-Mann-Whitney U-Test was used for pre/post analysis. Correlational coefficients were calculated by linear regression analysis. A p-value of less than 0.05 was considered statistically significant.

Results

Plasma glutathione levels and form

Baseline (day 1) total plasma glutathione levels of HIV-infected patients were lower than reference values obtained from healthy blood donors (1.95 ± 0.8 µM vs. 2.64 ± 0.74 µM, p = 0.03). After 2 weeks of therapy (day 15) total plasma glutathione levels were 2.51 ± 1.48 µM in the Immunocal group (+ 24.5 ± 59 %, p = 0.43), and 2.79 ± 1.2 µM in the Protectamin group (+ 44 ± 56 %, p = 0.004). The absolute change from baseline to day 15 was similar in both treatment groups (Immunocal: +0.42 ± 1.2 M, Protectamin: +0.77 ± 0.9 M, p = 0.84). Furthermore, mean total glutathione plasma concentrations were elevated during the whole study period (day 1 (n = 30): 1.95 ± 0.8 µM; day 15 (n = 29): 2.74 ± 1.2 µM, p= 0.01; month 1 (n = 18): 2.18 ± 0.9 µM, p = 0.19; month 3 (n = 16): 2.39 ± 0.9 µM, p = 0.06; month 6 (n =13): 2.47 ± 0.8 µM; p = 0.03, all comparisons with baseline, Fig. 1). The subanalysis of plasma GSH levels of 18 patients who completed both phases of the trial showed similar results without reaching statistical significance (day 1 (n = 18): 2.07 ± 0.8 µM; day 15 (n = 18): 2.74 ±1.2, p = 0.03; month 1 (n = 18): 2.18 ± 0.9, p = 0.36; month 3 (n = 16): 2.39 ± 0.9, p = 0.16; month 6 (n = 13): 2.47 ± 0.8, p = 0.10; all comparisons with day 1). As expected, levels of oxidized glutathione were mostly near or beneath the lower limit of detection of the assay and were not included in the statistical analysis.

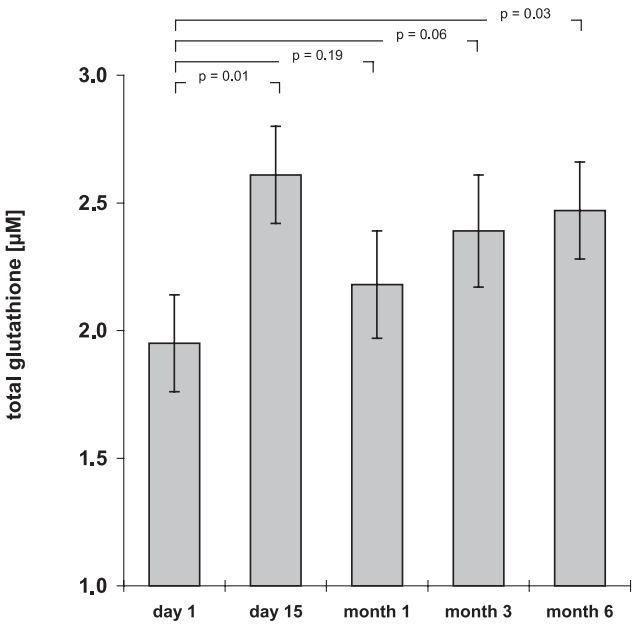

Fig. 1 Total plasma glutathione levels (mean ± SEM) were determined pre-therapy (day 1, n = 30), at the beginning of the follow-up (day 15, n = 29), after 1 month (month 1, n = 18), after 3 month (month 3, n = 16) and after 6 month follow-up treatment (month 6, n = 13) with whey proteins.

Clinical status

Body weight (base line: 71 ± 10 kg, month 1: 69.4 ± 9 kg, month 3: 73 ± 7 kg, month 6: 72 ± 8 kg), absolute CD4+ cell count (baseline: 243 ± 102 /µl, month 1: 263 ± 99 month 3: 286 ± 131, month 6: 256 ± 107 µl), CD4 / CD8 ratio (baseline: 0.35 ± 0.3; month 1: 0.31 ± 0.2, month 3: 0.31 ± 0.2, month 6: 0.33 ± 0.2) and routine laboratory parameters did not change in a significant way during the six-month course of supplementation.

Adverse events and compliance

A total of 26.6 % of patients receiving Immunocal and 60 % of patients receiving Protectamin reported adverse events or side effects during the pilot phase of the study. However, among these, only a few moderate side effects were potentially related to the study diet. Four patients complained about slight gastrointestinal disturbances which they related to the change in nutrition, and one patient in the Immunocal group discontinued the study due to gastrointestinal symptoms. There were no serious adverse events. No differences in frequency or severity of side effects were observed between treatment arms. Of the 18 patients who participated in the whole study period, 2 patients left the trial after three months due to gastrointestinal disturbance. In the remaining months, 3 more patients dropped out. One of them moved and two

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complained about the time-consuming preparation procedure of the whey nutriment. Thus, 5 of 18 patients (27.8 %) participating in the long-term trial quit the study prematurely. Eight patients reduced their daily dose because they felt that Protectamin had a negative effect on their appetite. As a consequence, the mean daily Protectamin intake was reduced from 45 ± 0 g/day during the pilot phase to 34.1 g/day in the remaining 13 subjects who completed the six-month study period. In total, 83 % of patients receiving Protectamin reported mostly mild adverse events. Only very few moderate adverse events were potentially related to the study diet, in particular gastrointestinal disturbances. There were no serious side effects.

Discussion

HIV infection is characterized by an increased oxidant burden and a systemic deficiency of the main antioxidant glutathione which occur early in the course of the disease, long before any clinical manifestations [4, 5]. This makes the glutathione system a potential target for adjuvant therapy in HIV-infected patients. The results of the clinical trial reported in this manuscript demonstrate for the first time that oral treatment of adult glutathione-deficient patients with advanced HIV-infection with a cysteine-rich nutritional supplement derived from whey proteins is able to significantly and consistently increase plasma glutathione levels into the normal range over a period of up to 6 months. Therapy was well tolerated; no clinically meaningful adverse effects were seen.

Several lines of evidence indicate that the glutathione deficiency may be of clinical relevance in the course of HIV infection [21, 23–27]. First, a beneficial effect of a therapeutic correction of the imbalance between oxidants and antioxidants has been demonstrated *in vitro* and *in vivo* [28, 29]. Second, intracellular glutathione levels of CD4+ lymphocytes predicted survival in HIV-infected individuals while restoration of intracellular glutathione levels by oral NAC increased survival rates of patients in a two year open-label follow-up study [17]. Third, in a recent double-blind study NAC treatment resulted in a significant improvement of immunological functions of NK-cells and T-cells [18]. The latter points in particular stress the clinical significance of the systemic glutathione deficiency in HIV infection, and the potential benefit of patients from a therapeutic intervention directed at augmentation of glutathione concentrations [15, 16].

Different strategies have been suggested to increase deficient glutathione concentrations in HIV-infected patients [13, 27, 30–32]. Theoretically, highly purified preparations of whey protein which contain large amounts of cysteine, glutamine, and glutamyl-cysteinyl are ideal dietary supplements to correct deficiencies of glutathione or cysteine, its precursor amino acid. There is even evidence that a mixture of small peptides as provided in whey proteins is of greater nutritive value than free amino acids with similar composition [29]. With this as background, this trial again [4, 17] confirmed that HIV-infected patients have plasma glutathione levels below the normal range which were significantly augmented by oral treatment with whey proteins. This is true despite the fact that only the results of the Protectamin arm reached statistical significance which is why Protectamin was chosen for the long-term study. However, these data do not allow the conclusion that Protectamin is superior to Immunocal. A direct comparison did not reveal a superiority of either preparation with respect to glutathione levels or any other parameter evaluated in this trial ($p = 0.84$) including compliance [21]. This feasibility study was neither designed nor powered enough to answer this question. Although a direct comparison of the two strategies is still lacking, effects of nutritional supplementation with whey proteins in HIV-infected patients on GSH levels were within the same order of magnitude as treatment with NAC. Based on other studies, it is tempting to speculate that the special amino acid composition of whey proteins could have additional beneficial effects on the immune system, on gastrointestinal homeostasis, and on stool frequency [33, 34]. Furthermore, whey proteins are virtually free of clinically relevant adverse effects or interactions with other medication, a point of particular relevance in HIV infection. It is therefore fair to conclude that whey proteins may be a therapeutic alternative to NAC in glutathione deficiency states. However, it is too preliminary to recommend NAC or whey proteins or another glutathione pro-drug as the treatment of choice for HIV-infected patients. Further studies in this respect are clearly warranted.

Importantly, open-label Protectamin normalized GSH-levels in the HIV-infected population over a period of 6 months with an even smaller daily intake of the whey protein formula compared with the pilot phase. A smaller amount of extra daily cysteine may be sufficient to keep glutathione levels within the normal range after initial restoration of the body cysteine reservoirs. The mean daily intake of 34.8 g whey protein represents approximately 800 mg of cysteine, only a fraction of the recommended total daily cysteine intake. However, this amount is well within the range of daily doses of N-acetylcysteine given in other clinical studies aimed at augmenting glutathione plasma levels [10, 13, 17, 18]. This trial basically confirms results from these studies both qualitatively and quantitatively.

Whey protein supplementation and the resulting increase of plasma glutathione levels did not translate into a clinically relevant treatment response during the treatment period. No significant improvement of parameters

relevant to patients with advanced HIV infection was seen. Future long-term studies of the effect of glutathione agonists such as whey proteins in HIV infection will also have to focus on parameters which are now recognized to be prognostically relevant (e. g., virus DNA plasma levels, body composition). In general, the study medication was well tolerated. However, oral supplementation of the daily diet of HIV-infected patients with 45 grams of whey proteins was more difficult than expected. Of 18 patients only 5 finished the trial still on the recommended daily dose of 45 g whey proteins. The main reasons for this discrepancy between the pilot phase and the long-term extension were gastrointestinal adverse effects, a negative effect on the patients' appetite, and the complex daily preparation procedure. All these factors affect compliance and directly reduce acceptance of a treatment in a group of patients with a normally high compliance. It remains to be seen if the whey protein formulation can be improved to compensate for these problems. Potential solutions include an enrichment of the cysteine content of the whey formulations to reduce the daily dose without reducing the active ingredient.

In conclusion, this preliminary study shows that whey protein formulas are able to increase glutathione levels in HIV-infected individuals, and are therefore a potential alternative to conventional glutathione prodrugs such as NAC. Up to now, nearly all evidence in favor of glutathione augmentation therapy in diseases characterized by glutathione deficiency is based on either *in vitro* experiments, animal models, or short-term clinical trials. It remains yet to be seen if the "biochemical efficacy" of therapeutic strategies such as augmentation of glutathione levels by oral whey proteins translates into a more favorable course of the disease assessable by key markers of disease activity. A large long-term randomized controlled trial is clearly warranted to study the benefit of whey protein therapy on survival and the course of the disease. The results presented in this study support this conclusion and underline the importance of the selection of an appropriate dose and application form of whey proteins.

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