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EFFECT OF INHIBITION OF GLUTATHIONE SYNTHESIS ON THE IMMUNOGENICITY OF CAPTOPRIL AND CAPTOPRIL–PROTEIN CONJUGATES IN THE MOUSE

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Abstract—The effect of glutathione (GSH) depletion on the immunogenicity of captopril (CP) and D-penicillamine (PA) was investigated in C57 mouse. Depletion of GSH was by DL-buthionine sulfoximine (BSO), a potent and specific inhibitor of GSH synthesis, injected on the days of immunization of the drug/drug–protein conjugates. Chronic BSO pretreatment for 5 consecutive days, before and including the 4-day immunization period, caused tissue GSH depletion (liver 60%, kidney 71%, lung 14%, spleen 14% and whole blood 36%) in C57 mouse. After chronic administration of CP (270 μmol/kg, i.p. or i.m.) or CP–HSA (200 μg, i.p. or i.m.), IgG antibody response to CP-derived antigen was detected by an enzyme-linked immunosorbent assay (ELISA). The IgG antibody recognised CP-OVA but not OVA, and was inhibited by CP in other protein conjugated form, thus confirming its specificity to CP. Depletion of GSH by BSO caused an increase in the CP-specific IgG antibody titre when CP/CP–HSA was immunized through i.p. (with or without Freund's Complete Adjuvant) but not i.m. administration. In contrast, chronic administration of PA (270 μmol/kg, i.p.) or PA–HSA (200 μg, i.p.), with or without BSO pre-treatment, did not lead to detectable PA-specific IgG antibody. The results again illustrate the difference in the intrinsic immunogenicity between CP and PA, in that depletion of GSH increased the humoral (B lymphocyte) anti-CP responses but not anti-PA responses under the experimental conditions. These findings suggest that GSH status, apart from its effects on the disposition of CP/CP–protein conjugates, should be considered as an important determinant of both the immunological and toxicological response to CP.

Captopril (CP*) is an angiotensin-converting enzyme inhibitor used widely for the treatment of hypertension and congestive heart failure. A number of adverse reactions associated with CP are thought to have an underlying immunological mechanism [1–3], and it has been suggested that CP with its sulphydryl moiety, or one of its metabolites, may act as a hapten. This view was further substantiated by studies in which CP bound extensively, via a covalent disulphide linkage, to plasma proteins *in vitro* and *in vivo* [4]. The CP–plasma protein conjugates were sufficiently stable to induce a CP-specific IgG antibody response in rabbits [5, 6] and in patients receiving the drug [7], although CP covalently bound to plasma proteins can be dissociated by thiol–disulphide interaction with endogenous thiols such as glutathione (GSH) and cysteine (CYS) both *in vitro* and *in vivo* [4, 8].

Like CP, the disposition of D-penicillamine (PA) is also affected by the endogenous thiols [9]. The formation and dissociation of PA–plasma protein conjugates, as in the case of CP, occurred readily *in vitro* and *in vivo* and was greatly affected by endogenous thiols such as GSH and CYS. However, as shown in previous studies, the difference in immunogenicity of CP and PA does not relate to their difference in disposition but difference in their intrinsic immunogenicity [9]. In this study, the effect of GSH depletion on the immunogenicity of CP and PA was investigated by determining the specific anti-CP IgG response and anti-PA IgG response, respectively, in C57 mouse. Depletion of GSH was by pretreatment with DL-buthionine sulfoximine (BSO) which is a potent and specific inhibitor of γ-glutamylcysteine synthetase, the enzyme which catalyses the initial step of GSH synthesis [10].

* Abbreviations: BSA, bovine serum albumin; BSO, DL-buthionine sulfoximine; CP, captopril; PA, D-penicillamine; ELISA, enzyme-linked immunosorbent assay; FCA, Freund's Complete Adjuvant; OVA, ovalbumin; HSA, human serum albumin; GSH, reduced glutathione; CYS, cysteine.

MATERIALS AND METHODS

Reagents. Bovine serum albumin (BSA, fraction V), human serum albumin (HSA, fraction V), ovalbumin (OVA, grade V), D-penicillamine (PA), *O*-phenylenediamine dihydrochloride (OPD), Tween 20, Freund's Complete and Incomplete Adjuvants, (F.C.A., F.I.A.), glyoxalase I (Grade IV), methylglyoxal, DL-buthionine sulfoximine (BSO), morpholinopropane-sulfonic acid (MOPS) and other general reagents were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Captopril (CP) was provided by the Squibb Institute (New Brunswick, NJ, U.S.A.). Horseradish peroxidase-labeled goat anti-mouse IgG was from Serotec Ltd. ELISA microtitre plates were from Becton-Dickinson Ltd. Disulphide-linked conjugates of CP-protein and PA-protein were prepared and characterized as described previously [5].

Depletion of glutathione (GSH) by inhibition of its synthesis. Male C57 mice (25–30 g) were given free

access to food and water. GSH synthesis was inhibited by intraperitoneal (i.p.) administration of DL-buthionine sulfoximine (BSO, 8 mmol/kg). Control mice received saline only (i.p.). To enable GSH depletion during the whole immunization procedure in this experiment, BSO was administered for 5 consecutive days, prior to and on the days of immunization, at 4-weekly intervals over a period of at least 5 months. Tissue GSH concentrations were measured in randomly selected mice on the final day of drug administration during one of the series of immunization.

Determination of glutathione concentrations. Tissue GSH concentrations were determined, as previously described [8], by the glyoxalase I method which is specific for reduced GSH and not affected by the presence of other endogenous or exogenous sulphydryl compounds.

Immunization with drug/drug–protein conjugates in mice. D-Penicillamine (PA, 270 μmol/kg, i.p.), captopril (CP, 270 μmol/kg, i.p. and i.m., with or without Freund's Complete Adjuvant) and PA–HSA/CP–HSA conjugates (200 μg/kg, i.p. and i.m.; with or without Freund's Complete Adjuvant) were injected in 0.15 M NaCl into male C57 mice (25–30 g, 10 animals per group). Injections of the free drug (CP/PA) and the drug–protein conjugates (CP–HSA/PA–HSA) were administered on 4 consecutive days and on the same days as when BSO was administered, at 4-weekly intervals over a period of 6 months. Blood samples were obtained by heart puncture 2 weeks after the fifth or sixth series of injections. Serum was obtained from the clotted blood by centrifugation and used to detect specific anti-drug IgG.

Enzyme-linked immunosorbent assay (ELISA) for anti-CP IgG antibodies. Microtitre plates were coated overnight at 4° with CP–OVA or OVA (100 μg/mL in 0.05 M phosphate buffer, pH 7.2, 125 μL/well). On the following day, the plates were washed three times over a 2-min cycle in 0.15 M phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween), and then shaken dry. Mouse serum was serially diluted 5-fold down columns in PBS-Tween (dilutions 1/1000–1/15,625,000 in duplicate, 100 μL/well) and the plates incubated for 1 hr at room temperature. The plates were washed as before and 100 μL of horseradish peroxidase-labeled goat anti-mouse IgG (diluted 1/1000 in PBS-Tween) was added per well. Incubation was for a further 1 hr at room temperature. The plates were washed again and 100 μL of substrate solution containing 0.4 mg/mL of *O*-phenylenediamine dihydrochloride (OPD) and 0.1% hydrogen peroxide (30% w/v) in 0.15 M citrate-phosphate buffer, pH 5.0, was added to each well. The enzyme-substrate reaction was terminated after 10 min by adding 50 μL of 25% sulphuric acid. Absorbances were read at 490 nm by a dual wavelength automated plate reader (Dynatech MR 710) with the reference wavelength set at 630 nm.

Enzyme-linked immunosorbent assay (ELISA) for anti-PA IgG antibodies. Mouse anti-PA IgG antibodies were assayed by the same method used for detecting anti-CP IgG antibodies as described, except that wells were coated with PA–OVA in place of CP–OVA. Total IgG antibodies against PA–HSA/HSA were detected by coating the microtitre plates with PA–HSA and HSA, respectively.

Inhibition ELISA. A fixed dilution of antiserum was incubated overnight at 4° with a range of concentrations (1–1000 μg/mL) of inhibitors in PBS-Tween. Insoluble material was sedimented by centrifugation (2000 *g*, 5 min). Supernatant fractions were assayed in duplicate for IgG antibody activity by ELISA. IC_{50} concentrations were determined by using a range of drug-derived protein conjugates as inhibitors (1 μg/mL to 1 mg/mL final concentration) to inhibit the interaction between the drug–protein conjugates and the IgG antibody in the ELISA studies. IC_{50} concentrations refer to the concentrations of inhibitors which decreased the antigen–antibody interaction by 50% in the ELISA study.

RESULTS

Depletion of glutathione (GSH) by inhibition of synthesis

GSH synthesis was inhibited after chronic BSO administration as indicated by depletion of tissue GSH in the BSO-treated group compared to the controls (Table 1). The extent of GSH depletion was different among the tissues studied, with significant depletion in the kidney (71%), liver (60%) and whole blood (36%). The lung and spleen only showed slight GSH depletion by 14% after BSO administration. The animals were randomly selected from a large pool of animals given the same treatment, therefore the tissue GSH concentrations should reflect the general GSH status of the whole group on the days of immunization with drug/drug–protein conjugates.

Effect of GSH depletion on the immunogenicity of CP/CP–HSA conjugates

Chronic i.p. administration of CP (270 μmol/kg, in saline) produced an anti-CP IgG response (measured as anti-CP–OVA activity), detected by ELISA in the sera after the fifth series of monthly injections (Table 2). When the same dose of CP in Freund's Complete Adjuvant was used for the immunization, the anti-CP response was increased, giving an average of anti-CP–OVA/anti-OVA titre of 40/<10. Pretreatment with BSO did not significantly increase the IgG response in mice receiving similar doses of CP (i.p. in saline). However, when CP was immunized in F.C.A. (i.p.), there were two high responders in the BSO-pretreated group, with an average anti-CP–OVA/anti-OVA titre of 336/<10. When mice were administered CP (270 μmol/kg in saline) intramuscularly, no specific anti-CP IgG response was detected in the control group, but a slight response was detected in some animals in the BSO-treated group. The poor response in these groups of animals given i.m. injections of CP may somehow be related to the tolerance phenomenon as proposed by a previous study in the rat [12].

When CP–HSA was used to immunize the mice, both i.m. and i.p. administrations (in saline) produced a positive anti-CP IgG response, with a total of 4 and 5 responders, respectively. The magnitude of the IgG titres was similar in both groups, with average anti-CP–OVA/anti-OVA titres of 5945 ± 570/<10 for the i.p. route and 5264 ± 645/

Table 1. Total glutathione (GSH + GSSG) concentrations (μmol equiv./g tissue; μmol equiv./mL blood) after chronic administration of DL-buthionine sulfoximine (BSO, 8 mmol/kg, i.p.) for 5 consecutive days in C57 mouse

Tissue	Total GSH (μmol/equiv./g tissue)		
	Control	BSO-treated	% depletion
Liver	8.11 ± 1.01	3.28 ± 0.50*	60%
Kidney	3.97 ± 0.36	1.16 ± 0.11*	71%
Spleen	5.99 ± 0.77	5.17 ± 0.44	14%
Lung	3.37 ± 0.41	2.91 ± 0.25	14%
Blood	1.19 ± 0.25	0.76 ± 0.09*	36%

Results are mean ± SD of six animals. * $P < 0.001$ using Student's *t*-test.

Table 2. IgG antibody titres directed against CP–OVA/OVA in serum samples of C57 mice immunized with CP (270 μmol/kg) or CP–HSA (200 μg/kg), with or without pretreatment with DL-buthionine sulfoximine (BSO)

Treatment	IgG antibody titres (anti-CP–OVA/anti-OVA)	
	Control	BSO-pretreated
CP, i.p.	10/<10 (5/5)	15/<10 (2/5)
CP, i.p. in F.C.A.	40 ± 6/<10 (6/6)	336 ± 28/<10 (2/6)
CP, i.m.	<10/<10 (0/6)	10/<10 (4/6)
CP–HSA, i.p.	5945 ± 570/<10 (5/6)	†29007 ± 2233/<10 (5/6)
CP–HSA, i.p. in F.C.A.	17041 ± 2271/<10 (6/6)	*24100 ± 2754/<10 (6/6)
CP–HSA, i.m.	5264 ± 645/<10 (4/6)	4980 ± 697/<10 (4/6)

IgG titres are calculated as reciprocal of the serum dilution giving an optical density of 0.5. Only titres >10 are shown. Figures in parentheses indicate number of responders per group. Results are means ± SD. * $P < 0.01$, † $P < 0.001$ using Student's *t*-test.

<10 for the i.m. route. When CP–HSA was immunized with F.C.A. (i.p.), all the animals in this treatment group gave a positive IgG response, with an average anti-CP–OVA/anti-OVA titre of 17041 ± 2271/<10. After BSO pretreatment, the anti-CP IgG response in mice immunized with CP–HSA (i.p. in F.C.A.), measured as anti-CP–OVA/anti-OVA titre, was further increased to 24100 ± 2754/<10.

Specificity of the anti-CP IgG response

The specificity of the IgG response for CP and CP–HSA, with or without BSO pretreatment, was determined by ELISA inhibition studies and the results shown in Figs 1 and 2, respectively. The anti-CP IgG activity in mice immunized with CP (i.p. in F.C.A.) was inhibited by CP-derived antigens such as CP–OVA ($IC_{50} = 3.2$ μg/mL), CP–HSA ($IC_{50} = 4.8$ μg/mL) and CP–BSA ($IC_{50} < 1$ μg/mL), but not inhibited by PA-derived antigens such as PA–OVA and PA–HSA. With BSO pre-treatment, the specific anti-CP IgG activity in mice pretreated with BSO and immunized with CP (i.p. in F.C.A.) was similarly inhibited by CP-derived antigens (CP–OVA, CP–HSA and CP–BSA) and not PA-derived antigens. The IC_{50} concentrations, however, were less than 1 μg/mL for all the CP-derived antigens.

The anti-CP IgG responses in mice immunized with CP–HSA (i.p. in F.C.A.), with or without BSO-pretreatment, were similarly inhibited by CP-derived

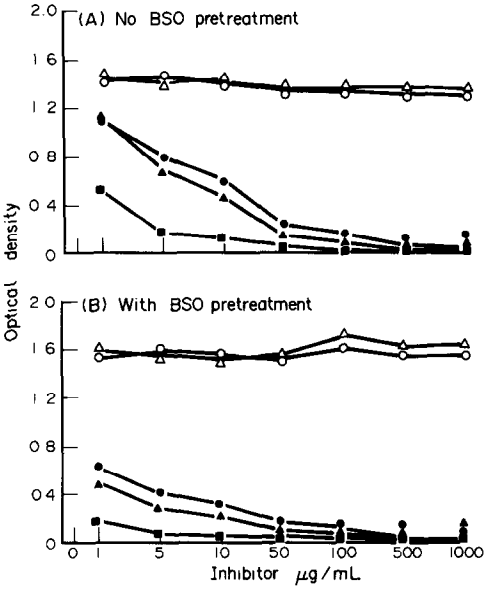

Fig. 1. Inhibition of captopril (CP)-specific IgG activity by preincubation of mouse antiserum, (A) without BSO pretreatment and (B) with BSO pretreatment (obtained after the fifth series of injections of 270 μmol/kg CP, i.p. in F.C.A.; 1/1000 dilution) with various concentrations of disulphide-linked drug–protein conjugates: CP-BSA (■), CP-OVA (▲), CP-HSA (●), PA-OVA (△) and PA-HSA (○). The CP-specific IgG activity was measured by ELISA.

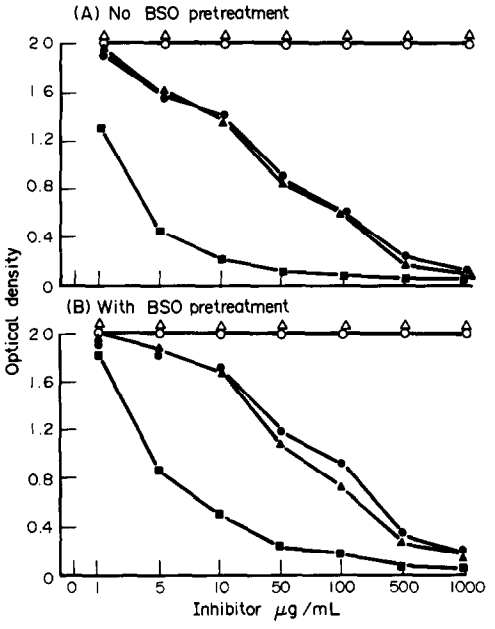

Fig. 2. Inhibition of captopril (CP)-specific IgG activity by preincubation of mouse antiserum. (A) without BSO pretreatment and (B) with BSO pretreatment (obtained after the fifth series of injections of 200 μg/kg CP-HSA, i.p. in F.C.A.; 1/1000 dilution) with various concentrations of disulphide-linked drug–protein conjugates: CP-BSA (■), CP-OVA (▲), CP-HSA (●), PA-OVA (△) and PA-HSA (○). The CP-specific IgG activity was measured by ELISA.

antigens (CP–OVA, CP–HSA and CP–BSA) and not by PA-derived antigens CPA–OVA, PA–HSA), again indicating the specificity of the anti-CP IgG activity (Fig. 2). The difference in the concentrations of the CP–protein conjugates (Table 3) required to inhibit the specific anti-CP IgG activity (IC_{50}) may be explained by the differences in the epitope density of the respective CP-derived antigens (CP–BSA > CP–OVA > CP–HSA). However, it is not possible to investigate whether BSO pre-treatment affected the qualitative aspects of antigen–antibody interaction in the experiment, although higher IC_{50} concentrations seemed to be required in samples of the BSO-pretreated group immunized with CP–HSA.

Effect of GSH depletion on the immunogenicity of PA/PA–HSA conjugates

There was no specific anti-PA IgG response, measured by anti-PA–OVA or anti-PA–HSA activity, after chronic i.p. administration of PA (270 μmol/kg, in saline), with or without BSO pretreatment (Table 4). When PA–HSA was used as the immunogen, only a non-drug-specific anti-HSA response was detected, by using both PA–HSA and HSA as test antigens, in both the BSO-treated and control group. The non-drug-specific anti-HSA titre was apparently increased by BSO pretreatment. When PA–OVA and OVA were used as the test antigens, only a weak drug-induced IgG activity was obtained in some responders in both groups (BSO-treated and controls). These responses, however, have high anti-OVA titres and were not significantly altered by BSO pre-treatment.

DISCUSSION

Sulphydryl drugs such as captopril (CP) and D-penicillamine (PA) are thought to be responsible for the hypersensitivity reactions in patients receiving the drug mainly because of the chemically reactive sulphydryl moiety in their structures, and thus having direct protein reactivity, although the prevalence of side effects of CP has since declined with low dose-regimens. The difference in immunogenicity between CP and PA has been demonstrated in previous studies [9]. Both drugs form conjugates with plasma proteins via disulphide linkages *in vitro* and *in vivo* [4, 9], and in spite of the short plasma half-life for CP-plasma protein conjugates of 1–1.5 hr [4], disulphide-linked CP-protein conjugates are immunogenic in humans and in animals [5, 7, 12]. Therefore, the potential of CP to function as a hapten is dependent on glutathione concentrations and renal function [8, 13].

Glutathione (GSH) is known to function both directly and indirectly in many biological processes including protein synthesis, amino acid transport, enzyme activity, metabolism and the protection of cells [14–16]. Perturbation of the GSH status can occur as a consequence of genetic defects associated with deficiencies of specific enzymes of GSH metabolism [17], certain physiological conditions such as diabetes and renal diseases [18–23]; and also chemical reactions with xenobiotics. In the case of CP, the

Table 3. Inhibition concentrations (IC_{50}) of various CP-derived antigens inhibiting the specific anti-CP IgG response in mice immunized with CP (270 μmol/kg) or CP–HSA (200 μg/kg), with or without pretreatment with DL-buthionine sulfoximine (BSO)

Treatment	Inhibition concentrations (IC_{50}) (μg/mL)		
	CP–BSA	CP–OVA	CP–HSA
CP, i.p. in F.C.A.	<1.0	3.2	4.8
CP, i.p. in F.C.A. + BSO	<1.0	1.0	<1.0
CP–HSA, i.p. in F.C.A.	1.5	22.0	24.0
CP–HSA, i.p. in F.C.A. + BSO	2.7	25.1	31.6

(Refer to methods for calculations of IC_{50} concentrations.)

Table 4. IgG antibody titres directed against PA–HSA/HSA or PA–OVA/OVA in serum samples of C57 mice immunized with PA or PA–HSA

Treatment	IgG antibody titres*	
	Anti-PA–HSA/anti-HSA	Anti-PA–OVA/anti-OVA
PA, 270 μmol/kg, i.p.	<10/<10	<10/<10
PA, 270 μmol/kg, i.p. + BSO	<10/<10	<10/<10
PA–HSA, 200 μg/kg, i.p.	62812 ± 5994/65092 ± 3878 (5/5)	21 ± 2/10 (4/5)
PA–HSA, 200 μg/kg, i.p. + BSO	82079 ± 5382/83461 ± 3059 (5/5)	16 ± 1/10 (2/5)

* Titres are calculated as reciprocal of the serum dilution giving an optical density of 0.5. Only titres >10 are shown. Figures in parentheses indicate number of responders per group. Results are means ± SD using Student's *t*-test.

covalent binding to plasma proteins was significantly increased after GSH depletion by BSO pretreatment [24]. To investigate whether or not the immunogenicity of CP may be altered likewise, the GSH status of mice was perturbed by BSO pretreatment in this study, while CP or CP–protein conjugates were used to immunize the animals. One of the major advantages in using BSO is its ability to deplete tissue GSH (particularly hepatic and renal GSH) without affecting other enzymes involved in xenobiotic biotransformation [25, 26] and without having any effect on protein synthesis or body temperature [27]. In this study BSO was injected on the same days as when the animals were immunized with drug/drug–protein conjugates. Given that the tissue GSH levels only recover 24–48 hr after cessation of BSO administration, it can be assumed that the animals would have a low GSH level while being immunized with the drug/drug–protein conjugate.

BSO was effective in decreasing the GSH levels in all tissues measured, but the decrease was modest in blood, lung and spleen. The lesser response in blood to BSO may reflect a slow turnover rate of GSH in red blood cells [28]. The lower response of GSH in the lung and spleen simply reflects the slower rate of GSH turnover in these tissues. The low GSH levels in the liver and kidney after chronic BSO administration, were more relevant to the disposition of CP/CP–protein conjugates at large.

The specific anti-CP IgG antibodies titres against CP and CP–HSA (i.p.) were increased after BSO treatment. Taking into account the dissociation of CP–protein conjugates involves both thiol–disulphide interaction and reduction of disulphide bonds by thiol-reductase *in vivo* [4, 29] a slower rate of dissociation of CP–protein conjugates or an increase in the conjugation of CP to proteins after GSH depletion is expected *in vivo*, leading to an accumulation of the CP–protein conjugates. The anti-CP IgG antibodies in this study is again highly specific for the disulphide-linked forms of CP. As previously proposed, a more extensive covalent binding of CP to plasma proteins after BSO treatment may provide a metabolic basis for CP to act as a hapten. This is confirmed in this study when, in the absence of a sufficiently high concentration of GSH, the potential of CP to act as a hapten was increased, by virtue of the likely accumulation of CP–protein conjugates.

Another important question is whether GSH depletion would affect the immune function of the animals *per se*, and thus affect the intrinsic immunogenicity of the drug/drug–protein conjugates in question. There is little doubt that modulation of intracellular GSH may indeed affect immune responsiveness and lymphocyte proliferation *in vitro* [30], but exogenous thiols may themselves enhance lymphocyte proliferation independently, even in a depleted state of GSH [31]. However, the intrinsic immunogenicity of PA/PA–HSA was not affected by GSH depletion in this study as both PA and PA–HSA treatment did not produce PA-specific IgG antibody response with or without BSO treatment. Unlike CP, the quantitative aspect of the immunochemical response of PA/PA–HSA was not affected by BSO either.

In conclusion, depletion of GSH by BSO pretreatment caused an increase in the CP-specific IgG antibody response to CP and CP–HSA. This may reflect changes in the disposition of CP–protein conjugates *in vivo* rather than the intrinsic immunogenicity of the haptens. This study further confirms that GSH status should be considered as an important determinant of both the pharmacological and the toxicological response to CP. Given that CP can produce immunomodulatory effects *in vitro* and in patients receiving CP [3, 32–34], the effect of GSH depletion on the immunomodulating effects of CP and other sulphydryl agents merits further investigation.

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