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Elevated Serum Cu in Hodgkin's Disease and Inhibitory Effects of Ceruloplasmin on Lymphocyte Response *In Vitro**

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Abstract—*Ceruloplasmin (CP) a constituent of normal serum, and serum copper were measured and found elevated in patients with Hodgkin's disease. The ratio between serum copper and serum CP was found to be relatively constant indicating that about 90% of the total serum copper is bound to CP in all stages of the disease and that this is not significantly different from normal.*

Patients with Hodgkin's disease usually have decreased immunoreactivity, it was therefore investigated whether the high CP content found in serum from these patients would be immunosuppressive.

In the present study, CP was found to markedly inhibit the in vitro *proliferative response of normal human peripheral blood lymphocytes stimulated with phytohemagglutinin (PHA), concanavalin A (con A) and purified protein derivative (PPD), and to inhibit the mixed lymphocyte culture response. Generation of cytotoxic killer cells during mixed lymphocyte cultures was likewise inhibited whereas killing by preformed cytotoxic T cells was not influenced.*

It was concluded that increased serum CP which was found in 36% of the Hodgkin sera may act immunosuppressive but the extent of such effects in vivo *can only be suggestively evaluated by means of measurements of the lymphocyte response* in vitro.

INTRODUCTION

CERULOPLASMIN (CP) is a constituent of normal serum which is moderately elevated in concentrations in the acute phase of inflammation. In pregnancy and after estrogen treatment, levels are similarly increased as well as in patients with Hodgkin's disease where very high values of CP have been reported [1].

Analyses of serum copper reflect the concentration in serum of CP [2]. Based on a relatively small number of analyses of serum copper and serum CP from patients with osteosarcoma [3], it was shown that the ratio of serum copper and serum CP is relatively constant when elevated values from patient sera are compared to normal. A similar evaluation between low and high serum values has been attempted in the present study.

Human serum CP is a blue copper-containing serum glycoprotein. The mol. wt obtained by Rydén [4] is 132,000 and the molecule contains 6 copper atoms (0.289% Cu) and about 30 carbohydrate residues, mainly mannose, galactose, fucose, glucosamine and sialic acid.

Besides its significant oxidase activity as a metalloglycoenzyme, CP assists the mobilization of iron from iron storage cells in the liver to the plasma and CP acts as a copper donor for cytochrome oxidase and other copper enzymes [5]. Another property of CP, which is probably not related to its copper content but rather to its glycoprotein nature is its immunosuppressive effect on lymphocyte response *in vitro* [6, 7].

A large number of serum proteins that suppress lymphocyte transformation *in vitro* have been suggested as important regulators of the immune response including alpha globulin [8], alphafoeto-globulin [9], $alpha_1$-acid

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glycoprotein [10] and also glycopeptides obtained from various serum glycoproteins [11]. We report here that CP inhibits expression of certain lymphocyte responses, including blast transformation induced both by mitogens and the mixed lymphocyte reaction and killer cell induction. CP is considered to be yet another alpha-globulin and acute phase reactant that interacts with lymphocyte responses *in vitro*, which may be of significance for the anergy of Hodgkin's disease.

MATERIALS AND METHODS

Serum samples

Obtained from patients with Hodgkin's disease admitted to the Radium Centre for Jutland and serum copper determinations by means of colorometric determination of the cupro complex with 2,9-dimethyl-4,7-diphenyl-1,10-phenantrolin [12].

Human ceruloplasmin

Obtained from AB Kabi, Stockholm, Sweden (lot BE 19353), extracted from Cohn fraction IV by chromatography on DEAE-sephadex; $E_{610\,nm}/E_{280\,nm}=0.026$. After affinity chromatography on concanavalin A–sepharose (conA–sepharose, Pharmacia, Uppsala, Sweden) and elution of the column according to Løwenstein [13] the purity of CP expressed by $E_{610\,nm}/E_{280\,nm}$ increased to 0.045.

Immunoelectrophoretic methods

Equipment and reagents were essentially as described in Weeke [14] 1% w/v Agarose gel (Litex, Glostrup, Denmark). Buffer: 0.04 M Tris, 0.04 M barbital buffer (pH 8.6 ± 0.1). Antibody: rabbit anti human ceruloplasmin, Dakpatts, Copenhagen). The rocket immunoelectrophoresis for quantitative CP determination was performed on 1.0 μl undiluted serum and known CP aliquots. Electrophoresis was performed for 18–20 hr at 10–12°C by a 2 V/cm gradient. Staining with coomassie blue 0.5%. Plates were photoprinted and the distances between the point of origin and the rocket tip were recorded in mm. A standard made of pooled normal serum was analysed in 18 consecutive series. Normal mean ± S.D. was found to be 39.4 ±2.9 mg/100 ml. Crossed immunoelectrophoresis for CP characterization was performed at 10 V/cm for 1.5 hr in the first dimension and 3 V/cm for 18 hr in the second dimension. The plates were pressed, washed and dried and stained as in rocket electrophoresis.

Blood separation techniques

Heparinized and sometimes glass bead defibrinated peripheral venous human blood was obtained from healthy male and female volunteers aged 22 to 43 yr. A total of 12 unrelated persons were used and all have previously been exposed to BCG. Mononuclear cells were obtained by centrifugation on Ficoll–Isopaque [15].

Lymphocyte culture

Reagents: medium 199 or RPMI 1640 (Gibco, N.Y., U.S.A.) containing 100 i.u. penicillin per ml was supplemented with heat inactivated normal human serum for standard culture medium. Phytohemagglutinin P. (PHA-P) (Difco, Detroit, MI, U.S.A.), concanavalin A (con A, Pharmacia, Uppsala, Sweden) and purified protein derivative (PPD) 50 i.u./mg batch RT-23 (State Serum Institute, Copenhagen, Denmark) were used as mitogens and antigens, respectively. Stock solutions were prepared in Dulbecco's phosphate buffered saline (PBS) at concentrations of 1–4 μg mitogen/ml and stored up to 2 months at 4°C.

After Ficoll–Isopaque separation the cell suspensions were washed twice in medium, counted and adjusted to 1×10^6 viable cells /ml culture medium. Test series using duplicate and triplicate cultures were set up in micro titer plates (Nunc, Roskilde, Denmark). Each culture contained $1\text{–}2\times10^5$ mononuclear cells in a volume of 0.2 ml. Mitogens and ceruloplasmin were added in μl quantities from the stock solution or diluted from these. Cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air of 37°C. At 18 hr before harvest 0.02 μCi of ^{14}C-thymidine (spec. act. 56 m Ci/mM) (Amersham, U.K.) was added to each well. The cultures were harvested as described elsewhere [16]. At least three successive test series were made. Results are presented as mean counts/min values, which normally differed from the individual ones by less than 10%. Mixed lymphocyte reactions were performed according to the method of Jørgensen and Lamm [16]. The assay used for detection of killer T cells generated after 6 days of mixed lymphocyte cultures was as described previously [17].

Cell viability before and during the culture period was checked by dye exclusion test. No toxic effect of CP was observed.

RESULTS

The relation between serum copper and serum CP

In Fig. 1, the results of 187 serum analyses from Hodgkin's patients with corresponding values of serum copper and serum CP are shown. A linear regression analysis on the total number of the analyses from normal values to extremely elevated values is seen in Table 1. The values are divided into two groups. Group I analyses within normal values ranging from 18.2 to 45.5 mg CP/100 ml serum and group II with CP values higher than 45.5 mg/100 ml. Crossed immunoelectrophoresis against anti human CP was done on the commercial product, the purified CP and sera from normal and patients with high CP values. Both single and tandem crossed immunoelectrophoresis were done for comparison between CP from different sources. In all cases a single peak with identical precipitation characteristics was seen from each CP source.

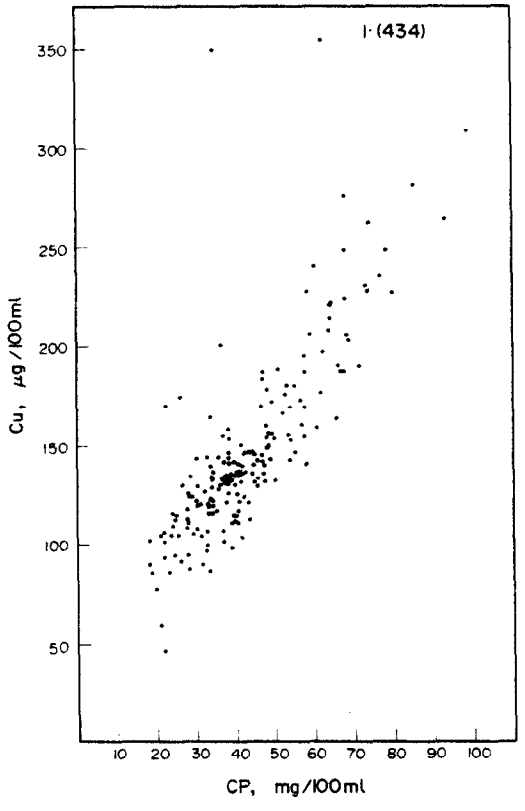

Fig. 1. Correlation between colorimetrically determined serum copper levels, µg Cu/100 ml *serum and serum ceruloplasmin levels, mg CP/100 ml serum, determined by rocket immunoelectrophoresis. One point showed very high serum* Cu *values.*

Table 1. Linear regression analyses and serum Cu/*CP values*

	n	SeCu*	SeCP*	*r*+	SeCu/SeCP
Normal	5	0.107	33.9	—	0.00315
Total group‡	187	0.149	43.2	0.79	0.00345
Group*	120	0.124	34.1	0.40	0.00364
Group+	67	0.193	39.4	0.72	0.00325

*Mean values (mg/100 ml serum).
+Correlation coefficient.
‡Sera from patients with Hodgkin's disease.

The effect of CP upon lymphocyte stimulation by con A and PHA

A series of experiments was designed to test the responsiveness of human lymphocytes to con A and PHA in the presence of varying amounts of CP. Figure 2 shows the inhibition of lymphocyte response after stimulation with 0.1 µg/ml con A and 0.1 µg/ml PHA. It is seen that 50% inhibition is obtained at about 50 µg CP/ml. Figure 3 shows the inhibiting effect of 0.5 mg/ml CP at varying doses of con A and PHA. Generally a tendency to higher inhibition by CP is noticed upon con A stimulation than after PHA lymphocyte

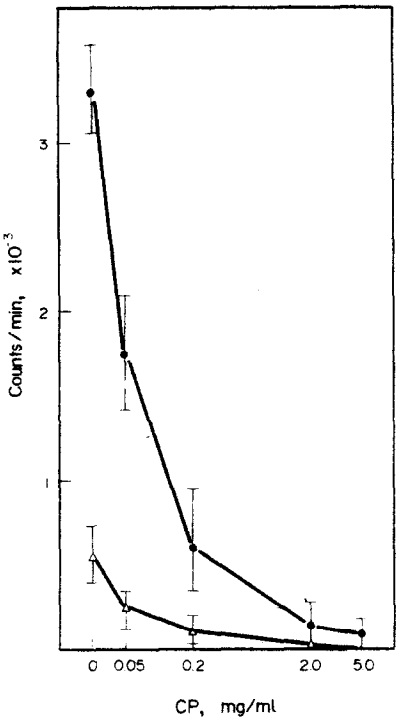

Fig. 2. Inhibition of PHA and con A induced stimulation of human lymphocyte cultures by simultaneous addition of ceruloplasmin (CP). (●) PHA 0.1 µg/ml; (△) *con A* 0.1 µg/ml. *Medium supplemented with 2% heat inactivated normal human serum.*

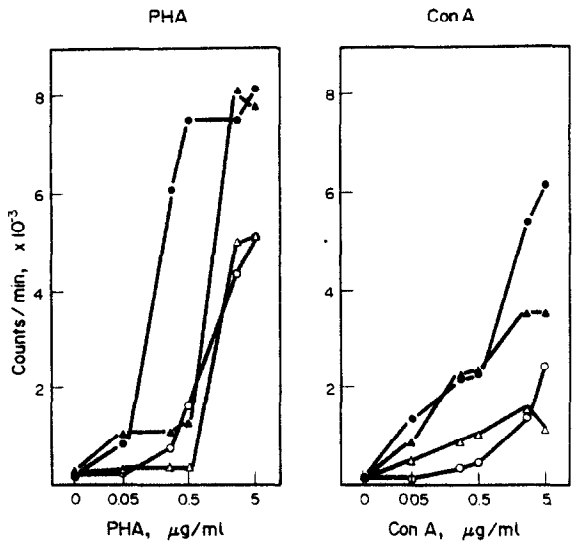

Fig. 3. Effect of ceruloplasmin on the PHA and con A dose–response of human lymphocytes measured at 2% and 18% serum concentration in the medium. (●) *2% serum;* (○) *2% serum +500 μg/ml CP;* (▲) *18% serum;* (△) *18% serum+500 μg/ml CP.*

stimulation. The influence of serum content in the incubation medium is also seen from Fig. 3. Lymphocyte response to PHA is higher at 2% serum content than at 18% for intermediate PHA concentrations whereas the response to con A at high serum concentration is decreased at the higher con A concentrations. The inhibitive effect of CP on PHA stimulated lymphocytes resembles the effect of high serum protein content. The effect of CP on con A stimulated lymphocytes is stronger and more inhibitive than for PHA.

The role of possibly direct interaction by CP with the cell surface was studied by means of preincubation for 2 hr and 24 hr with 500 μg CP/ml after which the cells were washed free of CP containing medium and transferred to fresh medium without CP but containing the mitogens. It was found (results not shown) that pretreatment for 2 hr had a variable effect whereas 24 hr preincubation had a consistently inhibitory effect. Cell viability after pretreatment judged by dye exclusion, was not affected as compared with cells pretreated in medium without CP. The inhibition in the subsequent culture obtained by pretreatment was never as pronounced as the inhibition observed in the presence of CP during the total culture period. Addition of CP at different days after initiation of PHA and con A stimulated cultures showed decreasing effect from day to day (Table 2). When present only during the last day of culture during which the thymidine uptake was assessed,

Table 2. Addition of CP at different days of culture

Mitogen*	Percentage of inhibition D.0†	D.1	D.2	D.3
PHA	84.7‡	54.4	23.8	4.3
Con A	72.5	75.4	48.5	27.5

*0.1 μg/ml.
†CP added to the lymphocyte cultures to a final concentration of 500 μg/ml at the time of mitogen addition (day zero).
‡Actual counts/min, mean of triplicate cultures. Control =12,487; addition of 500 μg/ml CP=1910.

minimal effect was seen which exclude trivial effects like impact on thymidine utilization.

Lymphocyte blastogenic stimulation by 0.2 μg PPD/ml was likewise inhibited 33–44% by 500 μg/ml CP. In some experiments DNA synthesis was measured at several time points during the culture period and the inhibitory effect of CP was found to be obvious at any point thus excluding shift in kinetics of the response to be responsible for the effects observed.

The effect of CP upon the mixed lymphocyte reaction (MLR)

The effect of CP was tested on human lymphocytes reacting in the MLR (Fig. 4). It is seen that about 75 μg CP/ml inhibits 50% of the response and that the inhibition of MLR by CP is comparable to the effect on

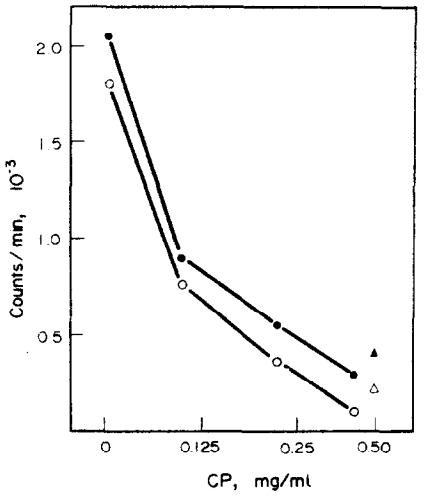

Fig. 4. Inhibitive effect of ceruloplasmin (CP) upon the mixed lymphocyte reaction (MLR) is shown in open circles (○). *For comparison the effect of CP on PHA stimulated lymphocyte response is shown in closed circles* (●). *PHA* 0.1 μg/ml, 2% *serum. CP used is purified,* $E_{610nm}/E_{280nm}=0.045$. *Effect of commercial CP,* 500 μg/ml, *is shown in triangles.* (△) *MLR,* (▲) *PHA stimulation.*

Table 3. CP induced inhibition of MLC and effector cell generation

	Thymidine ^{14}C incorp., counts/min (% inhibition)		Specific ^{51}Cr release* (% inhibition)	
Experiment	Control	CP 0.5 mg/ml	Control	CP 0.5 mg/ml
1	2495	1463 (41.4)	12.8	1.6 (87.5)
2	4005	1477 (63.1)	8.1	0.2 (98)

*Specific cytotoxic killer activity. Ratio killer:target = 20:1; 4-hr assay.

PHA stimulated cells under the test condition used in these experiments.

Effect on generation of cytotoxic killer cells

It was determined whether the inhibition of the MLR induced by the interaction with CP was only an effect on DNA synthesis or if one of the functional products of the reaction namely the generation of alloreactive cytotoxic cells was also influenced. This was accomplished by the addition of CP to MLR's, and the cells recovered after 6 days of culture were tested for cytotoxic activities against target cells from the person used as stimulator in a ^{51}Cr release assay [17]. In two experiments shown in Table 3, the thymidine incorporation in the control cultures not added CP was 2495 counts/min and 4005 counts/min, respectively. Addition of 500 μg/ml CP resulted in a 41% and 63% inhibition. Specific cytotoxic killer activity measured by ^{51}Cr release showed that there was 88% and 98% inhibition, respectively of the CP containing cultures compared to the controls (Table 3). Killer cells were compared by the addition of the same number of viable MLR generated lymphocytes to a fixed number of target cells. The ratio killer:target in the experiments were 20:1. Addition of various amounts of CP (50–5000 μg/ml) to the test tubes during the killer assay only did not influence the cytotoxic activity and the spontaneous release from target cells alone was not influenced by CP. These latter observations again show that non-specific toxic effects of CP on lymphocytes are unlikely to explain inhibition by CP.

DISCUSSION

Our results shown in Fig. 1 and Table 1 show that the ratio of serum copper and CP is relatively constant, indicating that the ratio of CP bound to non-CP associated copper in serum is maintained within certain limits in the present material consisting of sera from patients representing different stages of Hodgkin's disease. Comparison of the SeCu/SeCP ratios of Table 1 with the copper content of purified CP, 0.289% [4] indicates that from 10–20% of the serum Cu in Hodgkin's disease is not bound to ceruloplasmin. Shifrine and Fisher [3] studying serum copper and CP in osteosarcoma and in sera from women receiving chemical contraceptives similarly found a relatively constant ratio between serum copper and CP under these circumstances not different from normal.

A more differentiated picture of the relationship between these values and the clinical stages of Hodgkin's disease has resulted in the application of serum copper and CP analysis in monitoring the course of the disease and the effect of therapy [18].

An attempt to study by means of tandem crossed immunoelectrophoresis if CP in sera from patients with Hodgkin's disease changes in quality relative to normal CP did not show any differences in electrophoretic mobility. This criterion, however, is as pointed out by Krøll [19] not sufficient to prove chemical identity.

The present study demonstrated that CP inhibited DNA synthesis of lymphocytes both in response to two T cell mitogens as well as allogeneic cells. The inhibition of a constant amount of CP: 500 μg/ml on lymphocyte response by different concentrations of mitogen and of low and high serum concentration was assessed in Fig. 3. First if the influence of serum amount added to the culture medium was studied without addition of CP, it was found that optimal response to PHA is reached when 0.5 μg PHA/ml is used and the serum content is 2%. When 18% serum is added to the culture medium, 2.5 μg PHA/ml is needed to reach the same degree of lymphocyte stimulation. This indicates a probable interaction between PHA and serum proteins as suggested by Chase [20] who postulated that only unbound PHA stimulates lymphocyte response. Addition of 500 μg CP to

culture medium containing 2% serum results in a lymphocyte response pattern similar to the pattern obtained with 18% serum and no additional CP in the culture medium. This might be explained if CP interacts with PHA in a fashion which decreases the mitogenic potency of PHA. Amlot and Unger [21] conclude in their study of the immunosuppressive effect of sera from patients with Hodgkin's disease that the mitogenic effect of PHA is blocked by immunosuppressive serum compounds present in the sera. The decrease in mitogenic response to con A in the presence of CP may similarly be due to interaction, rendering conA inactive as mitogen.

The inhibition by CP of MLR was found similar to that of PHA induced lymphocyte response. The amount of CP which inhibited 50% of the MLR was from 50 to 500 μg/ml, depending on test conditions which compare favourably with the results found by Chiu *et al.* [10] for alpha$_1$-acid glycoprotein which inhibits 50% of the MLR at a concentration of 30 μg/ml. C reactive proteins inhibit 50% at 40 μg/ml [22] and alpha-fetoprotein at 250 μg/ml [23].

The generation of alloreactive cytotoxic cells which is known to be another functional potential of the thymus-derived lymphocyte system was found to be highly sensitive to inhibitory action of CP. The efferent phase (the cytotoxic action) was resistant to inhibition of CP.

All together it has become evident from this study that the lymphocyte proliferation assays thought to be *in vitro* correlate to cell mediated immunity are easily inhibited by CP. With regard to Hodgkin's disease the increased amount of CP as well as of other glycoproteins [24] is most probably responsive for the inhibitive effect of sera from these patients on lymphocyte stimulation as described by Hersh and Oppenheim [25].

Our results also suggest that there are interactions between PHA or con A with serum components and CP. The inhibitory effect of CP on MLR and the subsequent killer product, however, indicate that in fact part of the immunosuppressive effect is due to interaction of immunosuppressive components with lymphocyte functions. However, it is uncertain to draw definite conclusions from *in vitro* lymphocyte response studies to evaluate the *in vivo* effect of such serum components.

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