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THIOL-INDUCED CROSSLINKING OF HUMAN BLOOD PROTEINS: IMPLICATIONS FOR TUMOR IMMUNITY.

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Abstract: Incubation of human albumin and/or serum with a dithiol compound caused crosslinking of proteins by an intermolecular disulfide exchange reaction. This process was inhibited by a number of oxidizing and SH blocking agents. Similar protein copolymers were formed during incubation of serum with human transplantable lung cancer tissue.

Human serum albumin is a single polypetide chain containing 17 disulfide bridges which under mild reducing conditions undergo an intermolecular disulfide exchange reaction [1]. In normal human plasma less than 2 percent of the total albumin exists in the form of a polymer and copolymer with some other blood proteins, chiefly immunoglobulins [2,3]. Albumin and some immunoglobulins (IgG) were recently shown to be bound by intermolecular disulfide bonds to cartilage in rheumatoid arthritis [4]. It has been reported that the most prominent molecule on the surface of certain human parasites is covalently bound host serum albumin [5]. The sperm surface has also been demonstrated to be coated with female IgG crosslinked via disulfide exchange[6]. In these two latter cases, the significance of this phenomenon may be in masking antigenic determinants, thus preventing recognition by the immune system.

It has been previously demonstrated that albumin polymerization and gelation can be induced *in vitro* by incubation with 2-mercaptoethanol at 37°C for 16 hs.[7] In this study we have shown that gelation of human serum albumin (HSA) can be brought about in minutes by incubation with dithiothreitol (DTT) in a concentration dependent manner. With a 3% solution of HSA the gelation reaction can be observed already at the DTT"concentration of 5 mM, and reaches plateau (about 20 min.) at concentrations of 45-50 mM. Quite unexpectedly, gelation on incubation with DTT

has occurred not only with HSA, but with human serum as well. In order to examine the extent of copolymerization of HSA with other proteins, aliquots of serum mixed with DTT were electrophoresed at various times during incubation before the onset of gelation. One mL. of human serum was mixed with 40μL. of 0.3 M DTT and incubated at 37°C. Aliquots of 100 μL. were diluted with 300 μL. of barbital buffer (pH 8.6), and 3 μL. of diluted samples were then electrophoresed on agarose plates (Helena Labs., U.S.A.) for 15 min. at 120 V. The protein fractions were visualized by staining with Amido Black. This experiment has shown that in about 30 min practically all serum proteins formed a crosslinked copolymer remaining on the application point of the agarose plate (**Figure 1**). Clearly then DTT has capacity to induce disulfide exchange reactions not only between HSA molecules but with other serum proteins as well.

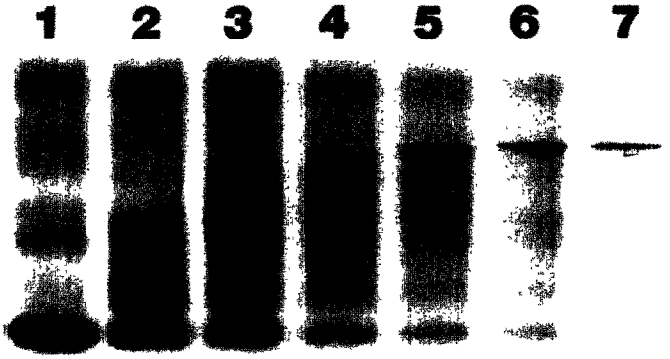

Figure 1. Time-course formation of protein polymers during incubation of human serum with DTT at 37°C. Samples incubated at 0, 5, 10, 15, 20, 25 and 30 min. are shown in lines 1-7 respectively.

To prove that the copolymerization reaction was a result of disulfide exchange, a number of substances known to interact with thiols were tested. Methylglyoxal was used as a compound known to react readily with SH groups [8]. Another method of inactivation of sulfhydryl groups is by oxidation to corresponding disulfides, and this was achieved by incubation in the presence of various oxidants. The effect of all these substances on gelation of human serum is shown in **Table 1.** The polymerization reaction was inhibited by methylglyoxal, ajoene, allyl disulfide, dehydroascorbic acid and cystine, but not by allyl sulfide, ascorbic acid or cysteine.

Table 1. Effect of various agents on gelation of human serum induced with DTT.

Normal human serum (320 µL.) was mixed with 0-80 µL. solutions of 0.1 M ascorbic acid, cysteine, freshly prepared cystine, dehydroascorbic acid and methylglyoxal adjusted to pH 7.4 with 1 N NaOH, and with 0.1 M ethanolic solutions of allyl sulfide, allyl disulfide and ajoene. After mixing with a solution of DTT (final concentration 20 mM) samples were incubated in glass test tubes at 37°C, and the onset of gelation was observed.

Agent	Inhibitory concentration*	Reactive group of the agent tested
Methylglyoxal	20 mM	-C(=O)-C(=O)-H
Ascorbic acid	>100 mM	-C(OH)=C(OH)-
Dehydroascorbic acid	20 mM	-C(=O)-C(=O)-
Cysteine	>200 mM	-SH
Cystine	20 mM	-S-S-
Allyl sulfide	>200 mM	-S-
Allyl disulfide	20 mM	-S-S-
Ajoene	10 mM	-S(=O)-S-S-

*Gelation time of serum was determined in triplicate. Standard error of determination was ±9%. All agents were tested at the concentration of 5-200 mM. Mean and ±SD for inactive substances was 15±3 min.(range: 11-23 min.).The reaction was considered inhibited when serum did not gel during 8 h.

Although it has been known before that albumin can copolymerize with other serum components, a phenomenon of crosslinking with practically all proteins has not been previously described. Such an efficient reaction is perhaps due to a specific structure of DTT, in which two thiols are separated by four carbons. It may be that this poly-thiol feature has relevance to biological systems. For example, high concentration of SH groups has been reported to exist on the surface of rapidly dividing cells, particularly cancer cells. The number of thiols on Ehrlich ascites tumor was calculated to be in the range of 10^7 per cell [9]. Gelation of HSA starts at a concentration of DTT of 5 mM, which is an order of magnitude higher than the molar concentration of HSA in the system tested. It would consequently appear that one tumor cell can initiate polymerization of about 10^6 molecules of albumin, and can induce crosslinking of a similar number of other serum proteins including immunoglobulins. In fact, incubation of HSA and serum with transplantable human lung carcinoma tissue resulted in the formation of a highly polymerized protein (**Figure 2**, lines 3 and 4).

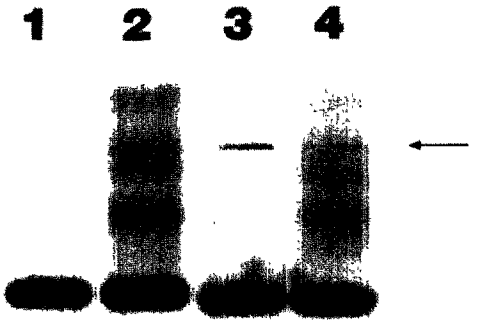

Figure 2. Formation of protein polymers during 18-h. incubation of HSA and human serum with a tissue of transplantable lung carcinoma (Lines 3 and 4). In addition to the appearance of high molecular weight polymers in samples incubated with tumor (arrow), diminished intensity of alpha, beta and gamma fractions in human serum is also noted (line 4 as compared to line 2). LX1 human lung carcinoma transplanted to Swiss BALB */nu /nu* mice was removed after the 15th day of growth. One g. of the tissue was excised, minced with a blade and washed with 10 mL. of deoxygenated normal saline. One hundred mg. portions of the tumor were mixed with 0.5 mL. of 3% HSA or human serum and incubated at 37°C for 10 min. and 18 hrs. After centrifugation at room temperature for 15 min. at 1,500x*g*, 3 µL. of the supernates were electrophoresed as described for Figure 1. Lines 1 and 2 are HSA and serum incubated for 10 min., and lines 3 and 4, for 18 hours respectively. High molecular weight protein polymers are shown as bands at the application points of the electropherograms (lines 3 and 4, arrow).

Many non-lymphoid tumors were shown to bind IgG via its Fc portion, and it has been suggested that this binding may mask surface antigens and block immune recognition and destruction of tumor cells[10]. We have recently proposed thatmalignant cells defend themselves against immune killing by forming a protective coat on their cell surface composed of crosslinked fibrin and/or fibrinogen resistant to plasmin degradation[11]. Inhibition of thiol-induced protein crosslinking by anumber of substances blocking or oxidizing SH groups suggests its siginificance in the *in vivo* situations. Consequently, methylglyoxal was documented to act as a cancerostatic agent[12]. Organosulfur compounds of garlic have been considered as potential anticancer agents[13,14] and ajoene, another cytotoxic compound isolated from garlic[15], is also a potential cancer chemoprotective substance[16]. It is also not unlikely that in the cases of cancer treatment with vitamin C [17],the active principle is dehydroascorbic acid which is readily formed in blood by oxidation of ascorbic acid[18].

Occasionally, gelation of serum occurred with freshly obtained tissue from tumors not older than 15 days, after incubation for at least two days. It is possible that high molecular weight protein polymers detectable in plasma of cancer patients by means of SDS-polyacrylamide gel electrophoresis[19], are formed *in vivo* by the thiol-induced intermolecular exchange reaction similar to that described in this paper.

In conclusion, thiol-induced crosslinking of human blood proteins *in vitro* may explain a mechanism by which tumor cells protect themselves against immune destruction. Blocking and/or oxidation of SH groups on the surface of malignant cells, which was shown in this study to inhibit protein crosslinking, may also be responsible for the therapeutic effectiveness of a number of naturally occurring substances.

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