EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Antioxidant Spectrum of Blood Serum and Its Peculiarities in Children

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We compared the contribution of high- and low-molecular-weight antioxidants into total antioxidant activity of blood serum in children and adults. Ten serum samples from children aged from 3 months to 12 years and 6 serum samples from adults were fractionated by chromatography and antioxidant activity and the contents of transferrin and ceruloplasmin were measured in total serum and individual chromatographic fractions. It was found that total antioxidant activity of the serum from children measured in the system of yolk lipoproteins considerably surpassed that in adults. Moreover, in adults the major part in serum antioxidant activity is played by a fist identified high-molecular-weight fraction (600 kDa) and a 67 kDa fraction containing ceruloplasmin and transferrin. Serum antioxidant activity in children was determined only by the high-molecular-weight peak not containing ceruloplasmin and transferrin, which was probably due to significantly lower serum transferrin content in children compared to adults.

Key Words: *children; blood serum; antioxidant activity; transferrin; ceruloplasmin*

Antioxidant activity (AOA) of the serum is determined by more than 15 components: vitamins E, C, A, β-carotene and other carotenoids, trace elements, enzymes, ion-chelating proteins, bilirubin, ureic acid, *etc* [4,6]. However, individual contribution of antioxidant components into total serum antioxidant activity was not determined.

Few reports on possible role of transferrin (TF) and ceruloplasmin (CP) in blood AOA are of particular interest in this respect [4,12]. It was found that serum content of TF and CP in infants is lower than in adults [8-10]. On the other hand, blood AOA in children is higher than in adults [11]. This necessitates comparative study of the contribution of low- and high-molecular serum components, TF, and CP into total serum AOA in children and adults.

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We fractionated blood serum by exclusion column chromatography and measured AOA (on the model of yolk lipoproteins, YLP) and the content of TF and CP in individual fractions with different molecular weight.

MATERIALS AND METHODS

We analyzed 16 serum samples: 6 samples from adults and 10 from bottle-fed infants of the first year of life and 3-12-year-old children with various pathologies. Fasting blood was taken during routine clinical examination from head viens (in infants) and ulnary vien (in children and adults).

Exclusion chromatography was performed on a Superose 6 column (1×30 cm) calibrated using standard water-soluble globulins. Blood serum (100 μl) was applied to the column.

Tris-HCl buffer (0.01 mol/liter, pH 7.5) containing 0.15 mol/liter NaCl and sodium azide was used

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as the eluent (elution rate 0.4 ml/min). UV detector REPPS-1M (λ=280 nm) was used as flow detector. Fractions (1 ml, 25 fractions in each experiment) were collected using FRAK-100 collector (Pharmacia).

Total AOA of blood serum was measured as described previously [3] with some modifications [7]. The method is based on inhibition of Fe^{2+}-dependent LPO of YLP in the presence of blood serum *in vitro* [3]. The intensity of LPO was evaluated spectrophotometrically (at 532 nm) by the content of TBA-reactive substances (calculated for MDA) [2,13]. Total AOA of the blood (in rel. units) was calculated as the ratio of A_{532} in the presence and absence of test serum.

Whole blood AOA was measured in 10-μl (for children) or 50-μl (for adults) blood samples. The results were calculated for the volume of 10 μl by the formula: $AOA_{10}=(AOA_{50})^{10/50}$[5].

AOA in serum fractions obtained after chromatography on Superose 6 was measured in 300-μl aliquots of each fraction as described above.

The content of TF and CP in blood serum and individual fractions was measured by radial immunodiffusion after Manchini using porcine antiserum against human TF and CP in 1% agarose gel prepared on 0.03 mol/liter veronal-medinal buffer (pH 8.6).

Reagents: Superose-6, agarose-M (Amersham Biotech Pharmacia), tris(hydroxymethyl)aminomethane (Fluca), water-soluble globulins (Serva standards), porcine antiserum against human TF and CP, and human serum with known concentration of TF and CP (Sevac). Other reagents (chemically-pure grade) were manufactured in Russia.

The data were processed statistically using Fisher dispersion analysis and Student parametric test [1].

RESULTS

The geometric means of total AOA of blood serum in children and adults were 2.45 (1.39; 4.30) and 1.26 (1.23; 1.28), respectively ($p<0.02$), *i.e.* serum AOA in children was significantly higher than in adults, which agrees with published data [11].

The AOA profile in adult serum was characterized by clear-cut maximum in fraction No. 13 (Fig. 1, *a*), which corresponded to a molecular weight of 585 kDa. Another peak was formed by fractions Nos. 15, 16, 17 and corresponded to a molecular weight of 67 kDa. Immunochemical analysis of TF and CP revealed the presence of TF in fractions Nos. 16 and 17 (Fig. 2, *a*), and CP in fractions Nos. 15, 16, and partially in fraction No. 17 (Fig. 2, *c*). Thus, neither TF, nor CP was found in fraction No. 13 exhibiting maximum AOA and this AOA was not associated with these proteins. At the same time, the additional peak of AOA in fractions No. 15, 16, and 17 of adult serum was probably determined by the presence of TF and CP.

Thus, AOA of adult serum is primarily associated with two peaks determined by a high-molecular-weight fraction with maximum AOA and containing no TF and CP and with a 67-kDa peak, whose AOA is determined by TF and CP.

In contrast to adult serum, children serum contained only one peak with maximum AOA corresponding to fraction No. 13, while the additional peak formed by fractions No. 15, 16, and 17 was absent (Fig. 1, *b*).

Similarly to adult serum, TF was present in fractions Nos. 16 and 17, and CP in fractions Nos. 15, 16, and 17, but not in fraction No. 13 (Fig. 3). It should

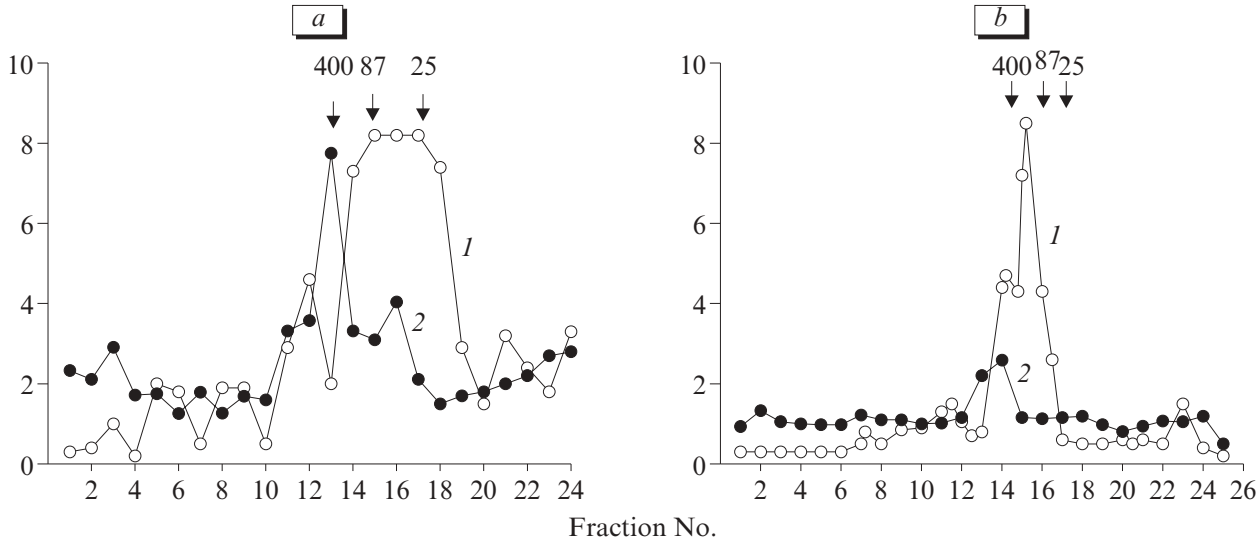

Fig. 1. Antioxidant activity (AOA) infraction obtained after chromatographic separation of adult (*a*) and children (*b*) serum on Superose 6 column. *1*) elution profile at 280 nm (opt. dens. units); *2*) AOA profile determined in YLP system (rel. units). Standard proteins are shown by arrows (440.87 and 25 kDa).

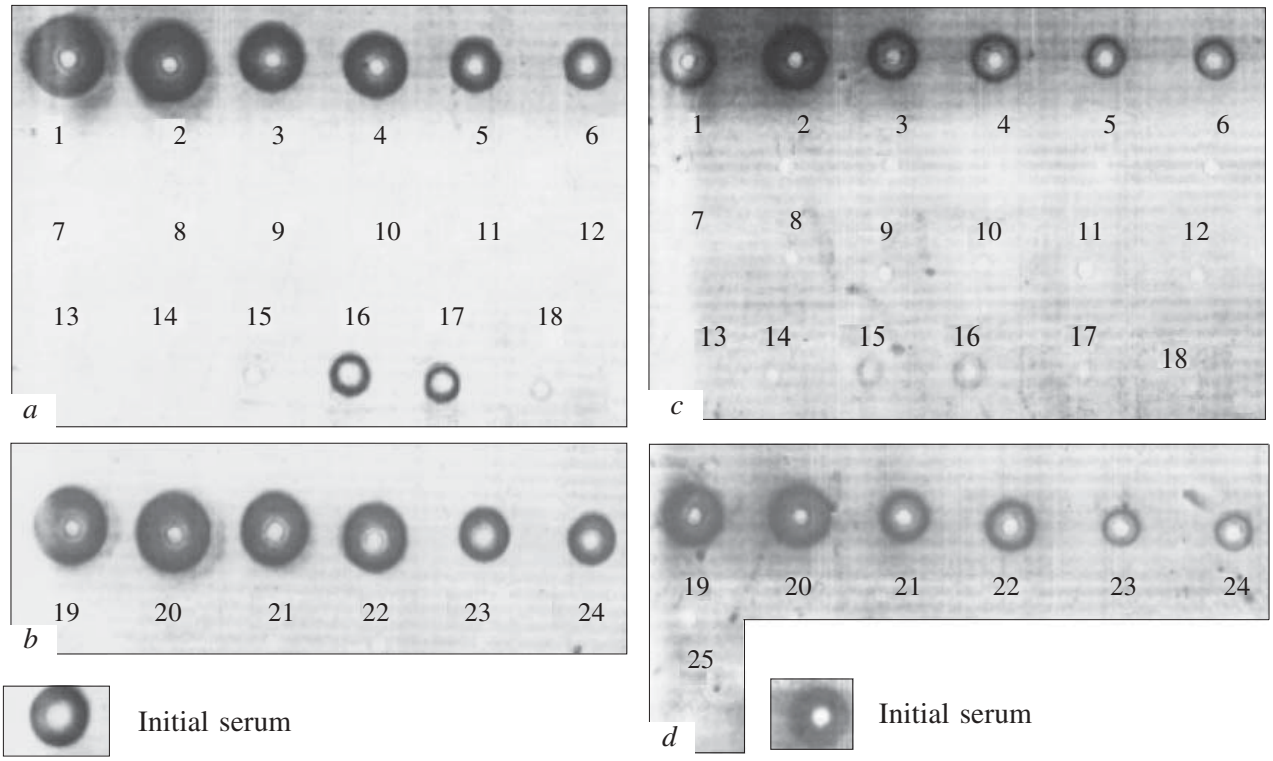

Fig. 2. Immunochemical analysis of transferrin (TF, *a, b*) and ceruloplasmin (CP, *c, d*) content in fraction obtained after chromatographic separation of adult serum. 1-25: fraction numbers; initial serum – TF (*b*) and CP (*d*) content in whole serum. First row: dilutions of standard serum against TF (*a, b*) and CP (*c, d*).

be noted that serum content TF in children was considerably lower than in adults (Table 1).

Thus, AOA of blood serum in children is primarily determined by the high-molecular-weight fraction (No. 13) first detected by us in the present study.

Attempts to characterize total serum AOA and the role of individual components were undertaken by I. Stocks *et al*. [12], and G. I. Klebanov *et al.* [4]. I. Stocks used brain homogenate as a model system and found that total AOA in adults is determined by serum proteins CP, TF, and albumin; vitamin E in physiological concentrations plays little role in total AOA, while other classical components of serum AOA (ascorbate, urate, carotenoids) have no effect on this model [12].

G. I. Klebanov *et al.* [4] also used YLP for measurements of AOA in fractions obtained after fractionation of adult serum on a TOYOPERL HW-60 column.

TABLE 1. Content of TF and CP in Children and Adult Serum (*M*±*SD*)

Protein, mg/dl	Children 0-12 years (*n*=10)	Adults, above 25 years (*n*=5)
TF	170.4±43.6	264.3±67.8*
CP	40.25±14.30	35.9±13.9

Note. $p<0.01$ compared to children.

It was found that serum AOA is primarily determined by CP present in the fraction of medium-molecular-weight proteins. At the same time, low-molecular-weight fractions have no effect on the parameters of the test system (chemiluminescence intensity in the system of YLP).

Both authors used chromatographic carriers not allowing effective fractionation of proteins with molecular weights above 200 kDa. We used Superose 6 allowing separation of proteins with molecular weights from 5 to 5000 kDa. This technique allowed detection of an additional peak with a molecular weight of ~600 kDa with maximum AOA. By its molecular weight this fraction approaches high-molecular-weight complexes (*e.g*. lipoproteins). It can be hypothesized that AOA is determined by some compounds (including low-molecular-weight substances) exhibiting AOA and bound to this complex (e.g., coenzyme Q, vitamin E). These substances in complex with other components of this fractions can effectively inhibit LPO in the Fe-YLP system. Analysis of this fraction will help to explain the differences in serum AOA between children and adults and the significance of this phenomenon.

Fractionation of blood serum under our experimental conditions revealed the absence of TF/CP-associated peak of AOA that in children (in contrast to

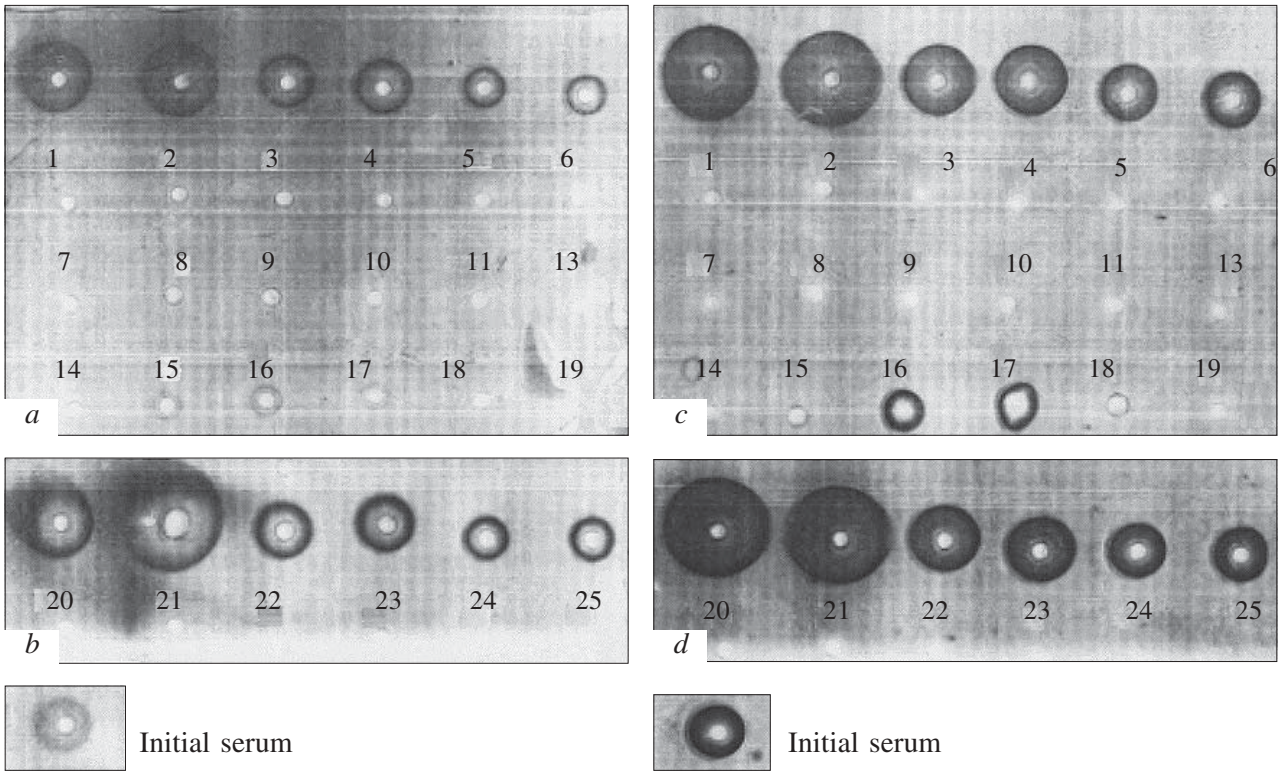

Fig. 3. Immunochemical analysis of ceruloplasmin (CP, *a, b*) and transferrin (TF, *c, d*) content in fraction obtained after chromatographic separation of infant serum. 1-25: fraction numbers; initial serum — TF and CP content in whole serum. First row: dilutions of standard serum against TF (*a, b*) and CP (*c, d*).

adults). This can be explained by lower content of TF in the serum of children compared to adults.

It was previously shown that low-molecular-weight components of the serum (ureic acid, ascorbic acid, and bilirubin) had no effect on parameters of YLP-based test systems [4]. At the same time, the concentration of these components of serum AOA is higher in newborns [11]. Therefore, complete characterization of the antioxidant status of children serum requires further studies directed at determining the contribution of individual components into total serum AOA with the use of alternative AOA recording systems allowing evaluation of the role of low-molecular-weight antioxidants.

It can be concluded that oxidative stress protection system in children and adults is determined by different antioxidants. This should be kept in mind during development and application of antioxidant therapy.

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