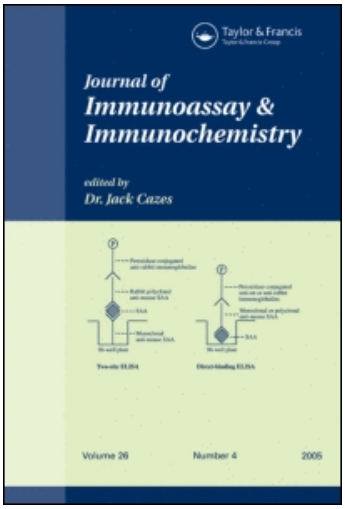

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Influence of Anticoagulants on the Level of Soluble HLA Class I and Class II Antigens Measured in Blood Samples

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INFLUENCE OF ANTICOAGULANTS ON THE LEVEL OF SOLUBLE HLA CLASS I AND CLASS II ANTIGENS MEASURED IN BLOOD SAMPLES

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ABSTRACT

The existence of soluble forms of HLA class I and class II antigens in human serum is well established and altered concentrations of these serum proteins have been described to be associated with various diseases. Since soluble HLA antigens (sHLA) can be measured both in serum and plasma samples, we investigated whether anticoagulant treatment influences the determined levels of soluble HLA class I (sHLA-I) or soluble HLA-DR (sHLA-DR). Analyzing paired samples of serum and plasma of 40 healthy individuals we found significantly lower serum levels of sHLA-DR (0.31 ± 0.15 ng/ml) compared to EDTA plasma levels (0.58 ± 0.20 ng/ml). By contrast, serum levels of sHLA-I (0.89 ± 0.74 μg/ml) were only slightly lower than EDTA plasma values (0.95 ± 0.86 μg/ml), a situation similar to that of sIL-2R and sCD4 levels. Further experiments intended to clarify the reasons of the reduced sHLA-DR serum levels revealed that (*i*) the blood storage time before centrifugation did not influence the sHLA-DR level, (*ii*) treatment of serum with anticoagulant did not augment the measured sHLA-DR concentration, and (*iii*) the recovery of spiked sHLA-DR was significantly lower when added to

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native blood than to serum or anticoagulant-treated blood. These results suggest that sHLA-DR is partly removed by the process of blood clotting thus resulting in diminished sHLA-DR serum levels.

(KEY WORDS: anticoagulants; ELISA; soluble HLA antigens)

INTRODUCTION

Human leukocyte antigens (HLA), encoded in the major histocompatibility complex (MHC), are membrane-bound glycoproteins which have the function to present antigenic peptides to T cells. Class I HLA antigens, consisting of a heavy α chain and β_2-microglobulin (β_2m), present endogenous peptides on the surface of a target cell to CD8-positive, cytotoxic T cells. By contrast, class II antigens, consisting of an α and a β chain of approximately the same size, predominantly present exogenous peptides to CD4-positive T-helper cells, thereby playing a key role in the initiation of an immune response.

More than two decades ago it was found that HLA antigens not only exist in membrane-bound but also in soluble form. These soluble HLA class I (sHLA-I) and class II (sHLA-II) antigens are present in human serum as originally determined by neutralizing allogeneic and xenogeneic HLA-specific antisera (1-3). Meanwhile, many reports described quantitation of soluble HLA class I (4-6) and class II (7-9) antigens in serum by ELISA, but only in the case of sHLA-I a commercial immunoassay is currently available (10).

Serum levels of sHLA-I are described to be a useful diagnostic parameter in infectious diseases and rejection episodes of transplants, as reviewed in (11). By

contrast, increased serum levels of sHLA-II were found in various autoimmune and leukemic diseases (12, 13), whereas no association with rejection of cardiac, liver and kidney transplants could be observed (14).

Here we report on the influence of various anticoagulants (EDTA, citrate, heparin) on the level of soluble HLA antigens measured in plasma compared to serum. The levels of sHLA-I as well as of soluble interleukin-2 receptor (sIL-2R) and soluble CD4 (sCD4) were found to be influenced only slightly. However, serum levels of soluble HLA-DR antigen (sHLA-DR) were found to be reduced to 50 % compared to plasma levels. We conclude that the process of blood clotting removes sHLA-DR from serum.

MATERIALS AND METHODS

Samples

Paired serum and plasma samples were prepared from native blood of 40 healthy individuals as follows: *Heparin plasma* was obtained by adding 10 µl heparin (10,000 IE/ml; Braun, Melsungen, Germany) to 10 ml of blood in a plastic tube, *EDTA plasma* was obtained by filling 7 ml of blood in an EDTA-coated tube (Greiner, Solingen, Germany) and *citrate plasma* was obtained by filling 8 ml of blood in a tube containing 2 ml of a ACD solution (acidum citricum + dextrose) (Greiner, Solingen, Germany). The addition of 2 ml ACD solution to 8 ml blood,

i.e. 4 ml plasma, caused a dilution of all plasma proteins to 66% and therefore the measured ACD plasma concentrations were corrected to the 1.5-fold value. *Serum* was obtained by incubating 10 ml of blood 4 hrs (unless otherwise mentioned) at 4°C to allow complete clotting. All samples were centrifuged (1,000 x g, 10 min), aliquoted, and stored at -25°C.

Antibodies

The HLA-DR-specific murine monoclonal antibodies L243 (15), obtained from ATCC (Rockville, MD), and BL-Ia/5 (16), kindly provided by Dr. I. Behn (Dept. of Biosciences, University Leipzig, Germany), were affinity chromatography purified from hybridoma culture supernatants using Protein A-Sepharose (Pharmacia, Uppsala, Sweden). L243 was biotinylated according to Boorsma *et al.* (17) using N-hydroxysuccinimidobiotin (Boehringer, Mannheim, Germany).

Quantitation of sHLA-I, sIL-2R and sCD4

Serum and plasma samples were analyzed for sHLA-I at a 1:25 dilution by means of the sHLA-STAT™ Class I immunoassay (SangStat Medical Corp., Menlo Park, CA). sIL-2R and sCD4 were quantitated in undiluted samples by use of Cellfree™ immunoassays (T Cell Diagnostics, Cambridge, MA) according to manufacturers' instructions.

Preparation of sHLA-DR Standard

To standardize the sHLA-DR immunoassay, described below, we used detergent-solubilized, affinity chromatography-purified HLA-DR from membranes of Balm 1 cells. HLA-DR purification was carried out according to Gorga *et al.* (18) with minor modifications. Briefly, Balm 1 cells were lysed in 0.5 % NP-40 lysis buffer containing a cocktail of protease inhibitors (PMSF, aprotinin, EDTA) for 30 min on ice. The lysate was cleared by centrifugation (3,000 x g, 30 min, 4°C) and the supernatant was depleted of IgG-reactive material (e.g. soluble Fc receptors) by passing through a human IgG-coupled Sepharose column (Sepharose 4B; Pharmacia, Uppsala, Sweden). Solubilized HLA-DR was then purified by loading on a L243-coupled Sepharose column. After washing with PBS and PBS containing 0.5 M NaCl the bound antigen was eluted by glycine-NaOH, pH 11.5. The eluted antigen was immediately neutralized by phosphate buffer, pH 4.5, and dialyzed against PBS. The protein concentration was determined (Proteinassay; BioRad, Hercules, CA) and the purity of the preparation was controlled by SDS-PAGE. For storage the sHLA-DR preparation was diluted with PBS containing 10 % dry milk to a concentration of 10 µg sHLA-DR per ml, aliquoted and stored at -80°C. This standard was stable for at least six month.

Quantitation of sHLA-DR

To quantitate sHLA-DR in serum and plasma we developed a double-

determinant enzyme immunoassay using the murine HLA-DR-specific monoclonal antibodies BL-Ia/5 and L243. In detail, a 96-well microtiter plate (Maxisorp; Nunc, Roskilde, Denmark) was coated with BL-Ia/5 (2.5 µg/ml in carbonate buffer, pH 10.6; 100 µl/well) overnight at 4°C. After washing three times with washing buffer (PBS containing 0.05% Tween 20) the plate was blocked with 300 µl/well blocking buffer (PBS containing 1 % dry milk) for 1 hr at room temperature. The blocking buffer was then aspirated and the samples were added at a 1:1 dilution by first filling 50 µl blocking buffer into each well and then adding 50 µl of the serum samples or the sHLA-DR standard (seven concentrations between 0.2 and 20 ng/ml sHLA-DR). After shaking five minutes the plate was incubated 90 min at 37°C. The plate was then washed three times, incubated with biotinylated L243 (1 µg/ml in blocking buffer, 100 µl/well) for 1 hr at room temperature, washed again three times and then incubated with peroxidase-coupled streptavidin (Dianova, Hamburg, Germany; 0.2 µg/ml in blocking buffer, 100 µl/well) for 30 min at room temperature. After finally washing six times, the substrate o-phenylenediamine (Sigma, St. Louis, MO; 340 µg/ml in citrate buffer, pH 5.0, containing 0.015 % H_2O_2, 100 µl/well) was added and the color was developed for 30 min in the dark. The reaction was stopped by addition of 50 µl/well 1 M sulfuric acid and the absorbency was measured at 492 nm.

This assay had an analytical sensitivity of 0.05 ng/ml sHLA-DR. Intraassay variations were below 10 % and interassay variations were below 15 % between 0.2 and 20 ng/ml sHLA-DR and in this range serial dilutions of different blood and plasma samples showed parallelism.

Recovery Rates

To determine the recovery rates of sHLA, serum/plasma or blood samples of known sHLA concentration [sample sHLA] were spiked with a fixed amount of purified sHLA [added sHLA]. After 4 hr incubation (and centrifugation of the blood samples) the sHLA concentration was measured by ELISA [measured sHLA] and the recovery was calculated as follows:

$$\text{recovery [\%]} = \frac{\text{[measured sHLA]}}{\text{[sample sHLA]} + \text{[added sHLA]}} \times 100\ \%$$

Statistical Analysis

Data were analyzed for a normal t-distribution by means of the Kolmogorov-Smirnov goodness-of-fit test. To look for significant differences between the groups the Wilcoxon signed rank test was used.

RESULTS

Levels of Soluble HLA Antigens in Serum and Plasma

Paired samples of serum, EDTA, ACD, and heparin plasma from 40 healthy individuals were assessed for their levels on soluble HLA antigens, revealing some

TABLE 1

Mean levels of sHLA-I, sHLA-DR, sIL-2R and sCD4 in serum and plasma of 40 healthy individuals (mean ± SD).

anticoagulant	sHLA-DR [ng/ml]	sHLA-I [µg/ml]	sIL-2R [U/ml]	sCD4 [U/ml]
EDTA	0.58 ± 0.20	0.95 ± 0.86	437 ± 181	12.0 ± 2.4
ACD	0.61 ± 0.24	0.98 ± 0.88	449 ± 150	11.8 ± 2.6
heparin	0.49 ± 0.17*	0.90 ± 0.80	439 ± 137	11.4 ± 2.2
none (serum)	0.31 ± 0.15*	0.89 ± 0.74	373 ± 124*	10.5 ± 2.0*

* significant difference to EDTA plasma ($p < 0.0001$, Wilcoxon signed rank test)

differences in sHLA-I and sHLA-DR (Table 1). sHLA-I levels, ranging between 0.2 and 3.5 µg/ml, were similar in plasma and serum. As expected, serum an plasma sHLA-I levels correlated at a high degree ($r > 0.95$) but no group showed a normal t-distribution, which is in accordance to previous reports (6, 19).

In contrast, sHLA-DR levels were significantly influenced by the presence and kind of anticoagulant used (Table 1). EDTA and ACD plasma showed the highest sHLA-DR concentrations in the range of 0.2 to 1.3 ng/ml, whereas heparin plasma and serum levels were significantly ($p < 0.0001$) reduced. Most strikingly, serum sHLA-DR levels were found to be only about 50 % of EDTA or ACD plasma values, which suggested an influence of blood clotting on the sHLA-DR concentration. sHLA-DR levels in serum and plasma were also highly correlated

($r > 0.75$) and, unlike sHLA-I, all four groups showed a normal t-distribution. No correlation could be found between sHLA-I and sHLA-DR levels, neither in serum ($r = -0.003$) nor in plasma (EDTA: $r = 0.090$; ACD: $r = 0.033$; heparin: $r = -0.027$).

Two other examined soluble leukocyte antigens, sIL-2R and sCD4 behaved like sHLA-I, i.e. serum and plasma values were comparable, however, the serum values were slightly but significantly ($p < 0.0001$) reduced to about 85-90% of the plasma values (Table 1).

The most striking result, the highly significant difference between sHLA-DR values measured in plasma (elevated levels) *vs.* serum (diminished levels) could be accounted for by several reasons: (*i*) the anticoagulant may induce sHLA-DR release from blood cells during the period of blood storage; (*ii*) the anticoagulant may strengthen the antigen/antibody reaction in the sHLA-DR immunoassay, thereby simulating an enhanced sHLA-DR concentration; (*iii*) during the process of blood clotting sHLA-DR may be removed from the serum by adsorption to clotting products. These possibilities should be confirmed or excluded by the following investigations.

Variation of Blood Storage Time

As depicted in Fig.1, prolongation of the blood storage time up to 8 hrs did not significantly change the sHLA-I or sHLA-DR concentrations. This argues against a measurable release of HLA antigens from blood cells during the storage

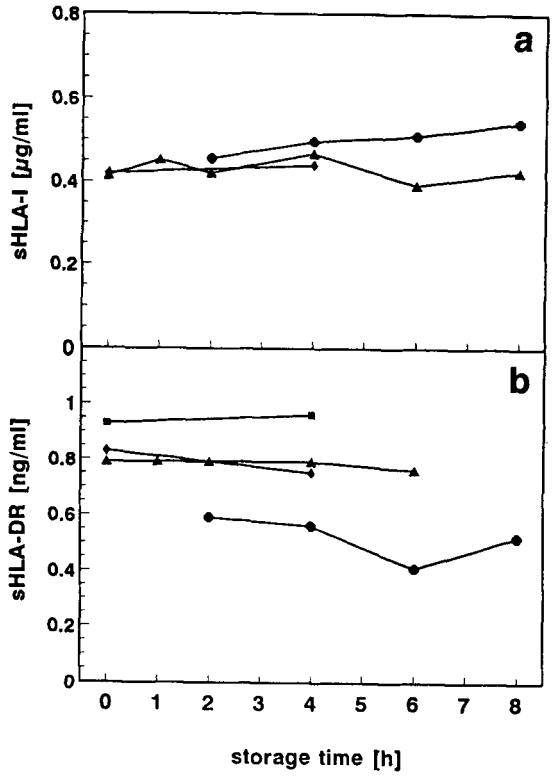

FIGURE 1. Influence of the blood storage time until centrifugation on the level of sHLA-I (a) and sHLA-DR (b) in EDTA plasma (▲), ACD plasma (■), heparin plasma (♦), and serum (●).

time until centrifugation. Similar results were obtained for sIL-2R and sCD4 concentrations (not shown).

Anticoagulant Treatment of Serum

Serum obtained from native blood of a healthy individual was subsequently treated with the anticoagulants EDTA, ACD, or heparin. The sHLA

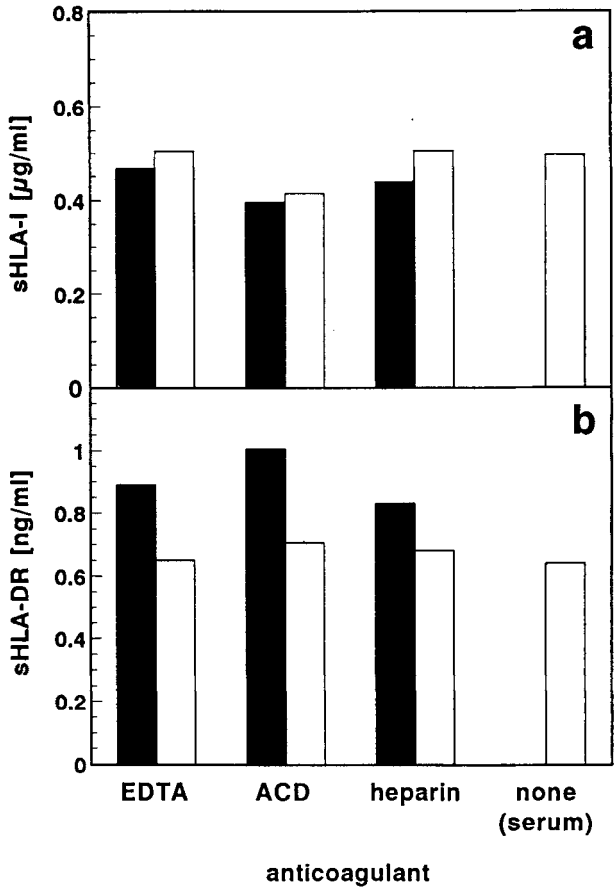

FIGURE 2. Concentration of sHLA-I (a) and sHLA-DR (b) resulting from blood treated with anticoagulants without clotting (plasma, gray columns) or from serum treated with anticoagulants after clotting (anticoagulant-treated serum, white columns).

concentrations of these samples (Fig.2, white columns) were compared with the corresponding plasma concentrations (gray columns). As shown in Fig.2b anticoagulant treatment of serum, i.e., after completion of the clotting process, did not elevate the serum sHLA-DR concentration to reach the respective plasma

level. Also serum sHLA-I (Fig.2a), sIL-2R, and sCD4 (not shown) concentrations were not altered by subsequent addition of anticoagulants.

This suggests a reduction of the serum sHLA-DR concentration caused by the process of blood coagulation rather than an enhancement of the plasma sHLA-DR concentration by strengthening antigen/antibody interactions in the sHLA-DR immunoassay.

Recovery of sHLA Added to Blood Samples

To study directly the effect of blood clotting on the level of soluble HLA antigens, we added a fixed amount of sHLA-I (100 ng to 1 ml) or sHLA-DR (3 ng to 1 ml) to untreated or anticoagulant-treated blood immediately after blood drawing. After an incubation time of 4 hrs the blood was centrifuged and serum/plasma concentrations of sHLA were measured to determine the recovery rates of the added soluble HLA (Fig.3, gray columns). These were compared to the recovery rates of samples, in which the soluble HLA was added directly to the serum/plasma, i.e., after centrifugation (white columns).

As shown in Fig.3b, the recovery rate of sHLA-DR was 69 % when added to native blood before the clotting process was initiated, as compared to 91 % when added to serum. This indicates that about 20 to 30 % of the spiked sHLA-DR was removed during blood coagulation. Interestingly, the recovery rates of sHLA-DR added to anticoagulant-treated blood were also slightly lower compared to those added to the corresponding plasma samples, suggesting an adsorption of

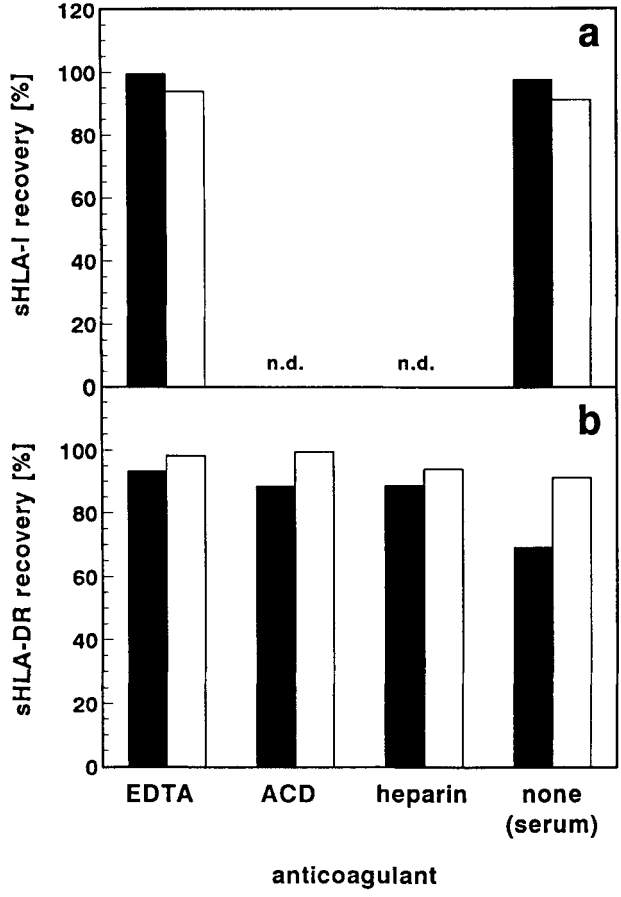

FIGURE 3. Recovery rates of sHLA-I (a) and sHLA-DR (b) resulting from blood spiked with sHLA before incubation and eventually clotting (spiked blood, gray columns) or from serum/plasma spiked with sHLA (spiked serum/plasma, white columns) (n.d. = not determined).

sHLA-DR to blood cells. By contrast, the recovery of sHLA-I was not found to be influenced by blood coagulation (Fig.3a).

DISCUSSION

There is growing evidence that soluble HLA antigens can be used as a diagnostic parameter in various diseases (11). For example, elevated sHLA-I levels were reported in infectious diseases (20, 21) and rejection episodes in various transplant recipients (22, 23), whereas elevated sHLA-II levels were found in patients with autoimmune (12) and leukemic diseases (8, 13).

In our study we measured levels of sHLA-I and sHLA-DR as well as sIL-2R and sCD4 in paired serum and plasma samples of 40 healthy individuals. Notably, in contrast to sHLA-DR, sIL-2R and sCD4, levels of sHLA-I were not normally distributed, indicating groups of "high secretors" and "low secretors" of sHLA-I, also described by others (6, 19). Our finding of a normal distribution of sHLA-DR levels is in contrast to a previous report (9) which describes also a population of healthy individuals secreting large amounts of sHLA-II. Also our measured sHLA-DR levels (0.31 ± 0.15 ng/ml) are three orders of magnitude lower than the sHLA-II concentrations reported by Westhoff *et al.* (9) (1.53 ± 2.43 μg/ml). These discrepancies may result from the different standards and the different antibodies used, e.g., we used a HLA-DR-specific antibody in contrast to a mixture of HLA-DR, -DQ and -DP-specific antibodies used by Westhoff *et al.*. Other reports on sHLA-II quantitation (8, 24) presented only values in "relative units", which are

not comparable to our data. In accordance with previous reports (9, 14) we did not find any correlation between sHLA-I and sHLA-DR serum or plasma levels.

With regard to the diagnostic potential of serum levels of soluble HLA antigens, we analyzed the influence of anticoagulants (EDTA, citrate, heparin) on the measurement of sHLA concentrations by ELISA technique.

Our main result was that in contrast to sHLA-I, sIL-2R and sCD4, the serum levels of sHLA-DR were reduced to about one half of the corresponding plasma levels. This finding is in accordance with another study (9), in which a 30 % reduction of sHLA-II levels in serum compared to EDTA plasma was reported. Three reasons might explain this surprising phenomenon: (*i*) the anticoagulants cause an enhanced release of sHLA-DR from blood leukocytes and/or a reduced degradation *ex vivo* during the storage period until centrifugation; (*ii*) the addition of anticoagulants alters the milieu in plasma, which strengthens the interaction between sHLA-DR and the anti-HLA-DR antibody during the immunoassay thus leading to an apparently higher sHLA-DR concentration in plasma samples; (*iii*) the process of blood clotting removes sHLA-DR from the serum resulting in a really decreased sHLA-DR concentration in serum samples.

The first possibility, an alteration of sHLA concentrations in the blood samples during the storage time before centrifugation, could not be confirmed. Such changes were described for levels of the cytokines interleukin (IL)-1, IL-6 and tumor necrosis factor-α and attributed either to a release by mononuclear cells induced by endotoxin-contaminated lithium heparin (25) or to an *ex vivo* degradation of the unstable cytokines (26). Interestingly, levels of IL-6, tumor

necrosis factor-α, and interferon-γ were also significantly lower in serum than in plasma (26). However, our observation that serum as well as plasma had unchanged sHLA-I and sHLA-DR levels, regardless whether the blood was stored two or eight hours before centrifugation, do not support the idea that *ex vivo* sHLA release or degradation significantly contributes to the differences between serum and plasma concentrations.

The second explanation could also be excluded: anticoagulant treatment of serum did not enhance the sHLA-DR levels to the corresponding plasma values.

Thus the third possibility seemed to be likely and could be supported by the finding that the recovery rate of sHLA-DR added to blood samples was reduced to about 70 % if the blood was allowed to clot, whereas anticoagulant-treated blood showed a recovery rate above 90 %.

In conclusion, we are tempting to speculate that plasma sHLA-DR is depleted during coagulation by adhering to fibrin clots. Additionally, some adherence to blood cells, e.g., to T cells by binding to CD4 and/or T cell receptor molecules, is suggested as small amounts of spiked sHLA-DR were also "lost" in anticoagulant-treated blood samples.

Besides the obvious reduction of sHLA-DR serum levels, there was also a slight but significant ($p < 0.0001$) reduction of sHLA-DR levels in heparin plasma, the reason of which remains unknown. Also serum levels of sIL-2R and sCD4 were found to be significantly ($p < 0.0001$) lower compared to their plasma levels, although they were reduced only to about 10 %. This suggests that blood clotting may also affect the concentration of these soluble surface antigens.

Summarizing, our data reveal that the presence or absence of an anticoagulant, as well as the kind of the anticoagulant might be important in the quantitation of soluble leukocyte surface antigens. Only samples treated with identical anticoagulants should be compared in further studies.

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