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Commercial Immunodepleted Deficient Plasmas Contain Cleaved High Molecular Weight Kininogen (HK)

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Key words

High molecular weight kininogen – Commercial, immunodepleted deficiency plasmas – Proteolytic events during immunodepletion of plasmas

Summary

Comparative analysis of high molecular weight kininogen (HK) in various commercial congenital and immunodepleted deficiency plasmas was performed by immunoblotting of HK. It was found, that some artificially depleted deficiency plasmas contained proteolytically cleaved, kinin-free kininogen. In contrast, in all congenitally deficient plasmas, HK was present in the intact, single chain form. Thus, cleavage of kininogen could have been triggered by or during the immunodepletion procedure. It was seen, that the degree of proteolytic cleavage and degradation of HK in depleted plasmas differed among various manufacturers. E.g. depleted products of one company contained only trace amounts of cleaved HK, in contrast to products of another one, in which HK was completely degraded. The immunoblot analysis of HK reflects the occurrence of proteolytic events during the production of artificially deficient plasmas and can therefore serve as a quality control method.

Introduction

In recent years, a need for plasmas artificially depleted of certain clotting factors developed for at least two reasons.

Firstly, some of the coagulation factor deficiencies are very rare, e.g. high-molecular-weight-kininogen (HK) (1, 2), prekallikrein (PK) (3), factor XI (4, 5), or factor VII (6) deficiency. This makes it increasingly difficult to collect sufficient amounts of congenitally deficient plasmas for coagulation assay purposes.

Secondly, due to repeated replacement therapy with coagulation factor concentrates, patients with clotting factor deficiencies, especially hemophiliacs, have been infected with HIV-I-virus (7, 8).

Thus, congenitally deficient plasmas have become potential health hazards. Therefore, one company performs steam-treatment of their lyophilized deficiency plasmas to eliminate possible risks (9).

Several other manufacturers began with the production of plasmas artificially depleted of clotting factors using HIV-antibody negative plasmas from tested healthy donors as starting material. From these normal plasmas one single clotting factor is selectively removed, generally by immunological methods. Usually, the immunodepletion-procedures are performed on immuno-affinity columns, where the plasma gets in contact with foreign surfaces (e.g. the column bed material or tubings). This surface contact can initiate activation of the intrinsic coagulation system via activation of coagulation factor XII (Hageman factor) (10, 11, 12). Activated F XII (F XIIa) enzymatically cleaves a number of substrates, such as prekallikrein and F XI (13, 14, 15) two components of the intrinsic clotting system. Prekallikrein is converted into kallikrein, an enzyme, which hydrolyzes kininogen and liberates bradykinin (Fig. 1) (16, 17). Thus, activation of F XII leads to proteolytic cleavage of kininogen. Therefore, the presence of cleaved HK is an indicator for the occurrence of proteolytic events, and the determination of HK in immunodepleted plasma is a valuable control of previous proteolysis.

In this study we tested various commercial deficiency plasmas of congenital origin as well as immunodepleted plasma samples for the presence of cleaved HK.

Materials and Methods

Test Samples

Congenitally and immunodepleted F VIII, F IX and protein C deficient plasmas were obtained from Behring AG, Marburg, FRG; Diagnostic Reagents Limited, Thame Oxon, England; Immuno AG, Vienna, Austria; Dade, Düdingen, Switzerland; Boehringer Mannheim, Mannheim, FRG; and Stago, Asnieres-sur-Seine, France.

All plasma samples were purchased in lyophilized form and were dissolved according to the manufacturers instructions. They were divided into aliquots and kept frozen at −20° C until use.

The samples were coded as shown in Table 1.

Reagents

Normal plasma and HK deficient plasma were obtained from George King, Biomedical Inc., Overland Park, KS.

HK-antibody. Polyclonal goat-anti-human kininogen antiserum (Nordic, Tilburg, Holland) preadsorbed as described below.

Binding of HK deficient plasma to NC-membranes. A 110 cm^2 sheet of a new NC-membrane was incubated with 7 ml PBS (containing 0.5 ml deficient plasma, 62 mg fibrinogen, 100 mM benzamidine, 20 mM NaN_3) for 2 hours at room temperature. After a brief washing step with 0.2% Tween 20, 20 mM NaN_3 in PBS, unoccupied binding sites on the membrane were blocked with dry milk. These membranes were used for preadsorption of HK-antibody solutions.

Preadsorption of HK-antibody. Anti-human HK antibody was diluted to a final concentration of 1.5% with either gelatine or non-fat dry milk solution (see Electroblotting procedure). An aliquot of 12.5 ml diluted antibody was incubated with 110 cm^2 nitro-cellulose membrane, to which HK deficient plasma was bound, for 4 hours at room temperature.

Purified, radiolabelled HK. HK was isolated from $Al(OH)_3$ adsorbed normal human plasma by sequential chromatography on QAE- and CM-Sephadex as described (17). The purified protein consisted of one band

Abbreviations

SDS – sodium dodecyl sulfate; PAA – polyacrylamide; RT – room temperature; NC – nitrocellulose.

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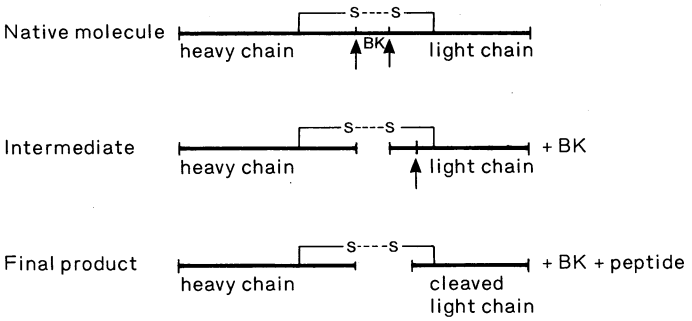

Fig. 1 Cleavage of high molecular weight kininogen by kallikrein (17): The arrows indicate the kallikrein cleavage sites

(MW 118 kDa) on reduced and two bands (MW 140 kDa, 118 kDa) on non reduced discontinuous SDS-PAA gels and it exhibited a specific clotting activity of 16 units/mg. Aliquots of the purified HK were radiolabelled with ^{125}I-Na (New England Nuclear, Dreieich, FRG) using a chloramine T-method (18). The labelled protein retained its clotting activity and its electrophoretic properties.

Nitrocellulose membranes (Hybond C) were purchased from Amersham (Amersham Place, England). All reagents were the best quality available.

Methods

Preparation of samples for electrophoresis. All plasma samples were diluted 1:10 with water before treatment either with 2% SDS (sodium dodecyl sulfate) or 2% SDS – 3.5% β-mercaptoethanol for 3 minutes at 95° C.

Activation of normal plasma with kaolin. Aliquots of 100 µl normal plasma were incubated with 16 µl kaolin (Fisher Scientific, 20 mg/ml saline) at 37° C for 30 minutes. The activated plasma was centrifuged for 30 seconds in a Beckman Microfuge B. The supernatant was discarded. The pellet was suspended in 50 µl PBS and this kaolin suspension was treated with 750 µl SDS or reducing agent as described above.

SDS-PAA gel electrophoresis. Was carried out on 7.5% SDS-PAA slab gels (150 × 150 × 1.5 mm) with a 4% stacking gel according to the method of Laemmli (19). Prestained molecular weight markers (BRL, Bethesda, MD) were included in each run. Electrophoresis was performed in a Hoefer-apparatus (Hoefer Scientific Instruments, San Francisco, CA) using constant current (40 mA/slab gel) and tap water cooling.

Electroblotting procedure (20, 21, 22). The electrophoretically separated samples were electrotransferred (electroblotted) onto nitrocellulose (NC) membranes using a Bio-Rad Trans-Blot system, filled with transfer buffer (25 mM Tris-190 mM glycine, pH 8.3). Blotting was usually performed at 4° C applying constant power (25 Watt/chamber) and a transfer time of 80 minutes. The NC sheets were quenched for 4 hours at RT with 0.4% gelatine-0.2% Tween 20, 20 mM NaN_3 in phosphate buffered saline pH 7.4 (PBS) or with 10% nonfat dry milk in PBS containing 1 mg/l thimerosal, 20 mM NaN_3, 0.01% Antifoam A and 1 µM phenyl-methyl-sulfonyl-fluoride, to block the unoccupied protein binding sites. Incubation with preadsorbed primary antibody followed in the next step.

The electro-blots were incubated in the preadsorbed 1.5% antibody solution for 7 hours at RT with constant agitation on a shaker (Red Rocker, Hoefer Scientific). After three 15 minutes wash cycles with 0.2% Tween, 20 mM NaN_3 in PBS, the NC membranes were reacted overnight with ^{125}I-HK [adjusted with blocking solution to 4 × 10^5 cpm/ml as described by Lämmle et al. (23)]. Unbound ^{125}I-HK was removed by four 10 minutes wash cycles with 0.8% Tween-0.5 M NaCl-20 mM NaN_3 in PBS, followed by three 5 minutes washes with 0.2% Tween, 20 mM NaN_3 in PBS. In some experiments treatment of the blots with the first antibody was followed by overnight-incubation with a biotinylated second antibody (rabbit-anti-goat immunoglobulin, Vector Laboratories, Burlingame, CA). The second antibody was used in a concentration of 0.02% in dry milk. Excess second antibody was removed by three 15 minutes wash cycles with 0.2% Tween-20 mM NaN_3. Bound biotinylated antibody was detected by incubation with ^{125}I-streptavidin (Amersham, Amersham Place, UK) for 2 hrs at room temperature. Radioactivity was adjusted to 4 × 10^5 cpm/ml blocking solution. Free ^{125}I-streptavidin was removed by washes with 0.8% Tween and 0.2% Tween as described above.

Bound radioactivity was detected by autoradiography.

Determination of HK clotting activity. Coagulation activity was assayed using an aPTT-test with hereditary deficient plasma as substrate. Briefly, kaolin, cephalin, deficient plasma and properly diluted test samples were incubated for 5 minutes. Coagulation was initiated by recalcification with 25 mM $CaCl_2$ and the clotting time was recorded.

Results

Coagulation Assays (Table 2)

H-Kininogen clotting activity is in the normal range for most samples tested. The F VIII and F IX deficient plasmas I, L, and E exhibit slightly reduced H-Kininogen activity (0.6; 0.66; and 0.68 units). However, four test samples: K, h, f and g contain rather low HK clotting activity, 0.48; 0.4; 0.39 and 0.45 units/ml).

Table 2 High molecular weight kininogen clotting activity and cleavage

Sample	Clotting activity (U/ml)	Visually estimated degree of cleavage
A	0.78	uncleaved
B	0.85	total
C	0.87	uncleaved
D	1.03	uncleaved
E	0.68/0.70	uncleaved
F, e	0.75/0.77/0.83	uncleaved
G (Normal)	0.93	uncleaved
H	0.88	uncleaved
I, i	0.60	uncleaved
K, h	0.48/0.4	total
L	0.66/0.75	total
M	0.70	uncleaved
N	0.78	uncleaved
a	0.85	partial
b	0.98	partial
c	0.78	partial
d	1.50	partial
f	0.39	total
g	0.45	partial

Table 1 Analyzed deficient plasmas

	Factor VIII Congenital	Depleted	Factor IX Congenital	Depleted	Protein C Congenital	Depleted
Behring	A	–	–	K, h	–	a
Boehringer	–	f	–	g	–	–
Diagen	–	B	–	L	–	–
Immuno	E*, F, e	–	H*, I, i	–	–	b, c
Merz & Dade	C	D	M	N	–	–
Stago	–	–	–	–	–	d

* Steam treated deficient plasmas (STIM-D).

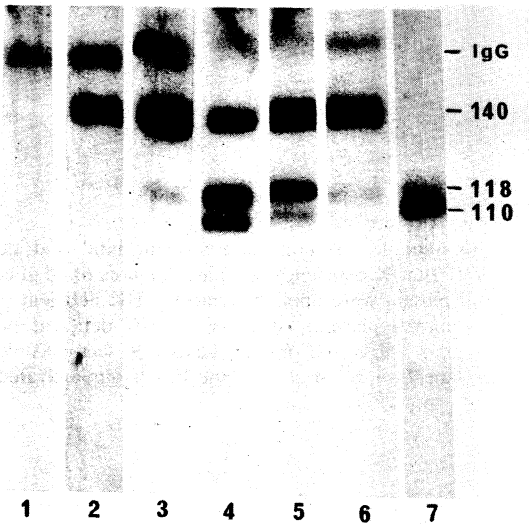

Fig. 2 Immunoblot of non reduced protein C deficiency plasmas. Aliquots of 40 µl containing 2.5 µl plasma were applied. Lane 1 – HK deficient plasma; lane 2 – normal plasma; lane 3 – normal human serum; lane 4 – sample a; lane 5 – sample b; lane 6 – sample d; lane 7 – kaolin activated HK

Immunoblotting Experiments

In our immunoblot system, non reduced samples of purified HK as well as plasma H-Kininogen migrate with a major band at a molecular weight of 140 kDa, and a minor band at MW 118 kDa as calculated from a calibration curve prepared with prestained molecular weight marker proteins (BRL) (Fig. 2, lane 2). Upon reduction, only one band with a MW of approximately 118 kDa can be identified (Fig. 5, lane 2). When native plasma HK is activated by the addition of kaolin via F XIIa and kallikrein (Fig. 1), the electrophoretic pattern of non reduced samples shows three bands corresponding to residual native HK (140 kDa) and two forms of activated HK (MW 118 kDa, 110 kDa). Reduction of activated samples leads to the appearence of two polypeptide chains on immunoblots, with MW of 58 kDa, corresponding to the intact light chain, and MW of 48 kDa representing the cleaved light chain of activated HK (Fig. 5, lane 1).

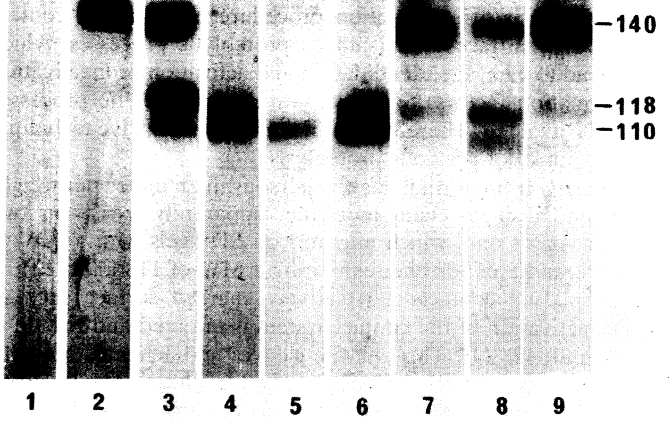

Fig. 3 Immunoblot of non reduced factor VIII and IX deficiency plasmas. Aliquots of 40 µl containing 2.5 µl plasma were applied. Lane 1 – HK deficient plasma; lane 2 – normal plasma; lane 3 – normal human serum; lane 4 – kaolin activated HK; lanes 5–7: F VIII deficient plasmas: lane 5 – f, 6 – B, 7 – e; lanes 8–9: F IX deficient plasmas: lane 8 – g, 9 – i

The heavy chain of activated H-Kininogen is not detected with the light chain specific antibody from Nordic.

Fig. 2 and 3 show immunoblotting results obtained after incubations with biotinylated second antibody and ^{125}I-streptavidin. Three non reduced protein C deficient plasma samples (lanes 4–6) are compared with normal plasma, kaolin activated normal plasma and serum.

All three protein C deficient plasmas contain partially activated HK as demonstrated by the presence of the 140 kDa, 118 kDa and 110 kDa bands. However, the degree of HK-cleavage varies, and is highest in sample a and lowest in sample d. In serum, H-Kininogen is present mainly in the intact form. This can be explained as follows: only part of H-Kininogen from blood is adsorbed and activated at a negatively charged surface (glass, kaolin), and part of HK remains in solution and intact. The adsorbed HK can be eluted e.g. with 2% SDS.

The eluate, for instance from kaolin, contains primarily cleaved H-Kininogen, as demonstrated by the presence of the 118 kDa and 110 kDa bands and the absence of the 140 kDa band (Fig. 2, lane 7).

Fig. 3 shows H-Kininogen in non reduced factor VIII and factor IX deficiency plasma samples in comparison with HK deficiency plasma, normal plasma, serum and the SDS-eluate from kaolin. The depleted F VIII deficiency plasmas f and B contain only cleaved HK in contrast to the congenitally F VIII deficient plasma sample e, where HK is present primarily in the intact form (140 kDa). The depleted F IX deficiency plasma g holds intact as well as cleaved H-Kininogen, whereas the congenitally F IX deficient plasma sample i contains only intact HK.

A band, migrating with a molecular weight of 160 kDa, which can be seen in all plasma samples, has been identified as IgG. The IgG band is only detected when the binding of the anti-H-Kininogen antibody to blotted H-Kininogen is visualized by incubation with biotinylated rabbit-anti-goat immunoglobulin, followed by ^{125}I-streptavidin. However, the presence of the IgG band does not hamper the determination of the kininogen cleavage. The use of a biotinylated second antibody and ^{125}I-streptavidin for the dedection of cleaved HK in plasma samples allows a wide application of the method, since it does not depend on the availability of purified, labelled H-Kininogen.

The results shown in Fig. 4 to 7 have been obtained with the antigen-overlay technique. Fig. 4 summarizes further results of congenitally and artificially deficient plasma samples using this highly specific technique. In all congenitally deficient plasmas (lanes 3–5: F VIII deficient plasmas E, F, C; lane 7: F IX deficient plasma sample i) HK is intact. Interestingly, in the depleted F VIII deficiency plasma sample D, HK is uncleaved, whereas in the depleted F IX deficiency plasmas, h and L, H-Kininogen is completely cleaved, and shows the same electrophoretic migration as the kaolin-eluate (lane 10) with a major band at 110 kDa and a minor band at 118 kDa.

Fig. 5 and 6 depict the immunoblotting results obtained with reduced F IX and protein C deficiency plasmas. For investigations of reduced plasma samples, the application of the antigen-overlay technique is required. In reduced samples IgG migrates as two bands with MW of 50 kDa and 23 kDa. A crossreaction of the biotinylated antibody and ^{125}I-streptavidin with plasma IgG interferes with the determination of HK cleavage via kininogen light chain (MW 48 kDa).

The depleted F IX deficiency plasmas h and L (Fig. 5, lanes 3 and 5) contain fully cleaved HK with a major band at 48 kDa.

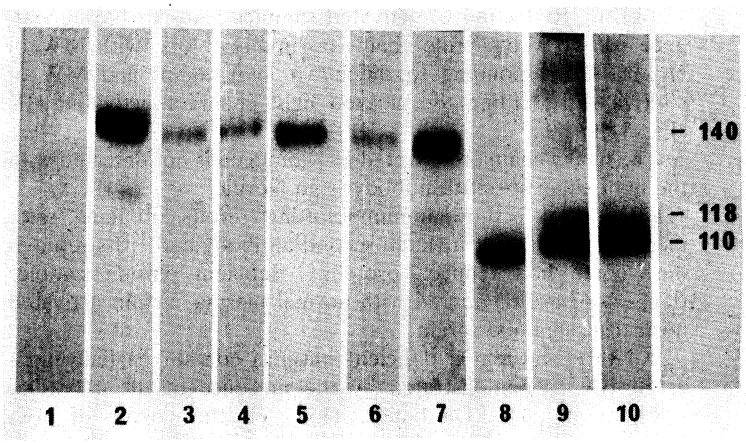

Fig. 4 Immunoblot of non reduced congenital and depleted factor VIII and IX deficiency plasmas. Aliquots of 40 µl containing 2.5 µl plasma were applied. Lane 1 – HK deficient plasma; lane 2 – normal plasma; lanes 3–6: F VIII deficient plasmas: lane 3 – E, 4 – F, 5 – C, 6 – D; lanes 7–9: factor IX deficient plasmas: lane 7 – i, 8 – h, 9 – L; lane 10 – kaolin activated HK

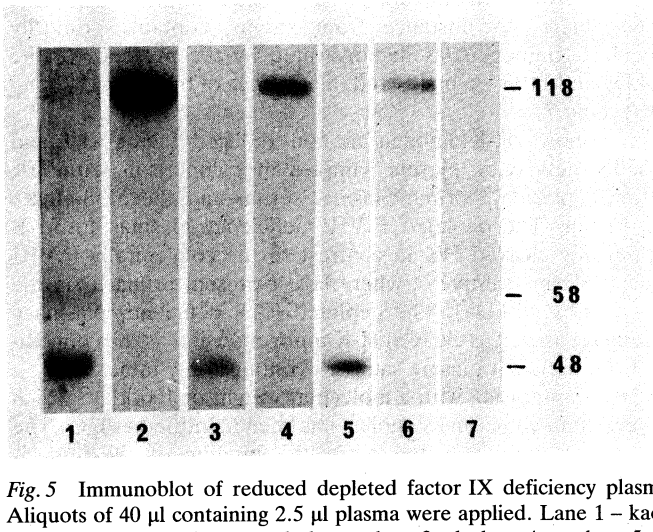

Fig. 5 Immunoblot of reduced depleted factor IX deficiency plasmas. Aliquots of 40 µl containing 2.5 µl plasma were applied. Lane 1 – kaolin activated HK; lane 2 – normal plasma; lane 3 – h; lane 4 – g; lane 5 – L; lane 6 – 0.1 µg purified HK; lane 7 – HK deficient plasma

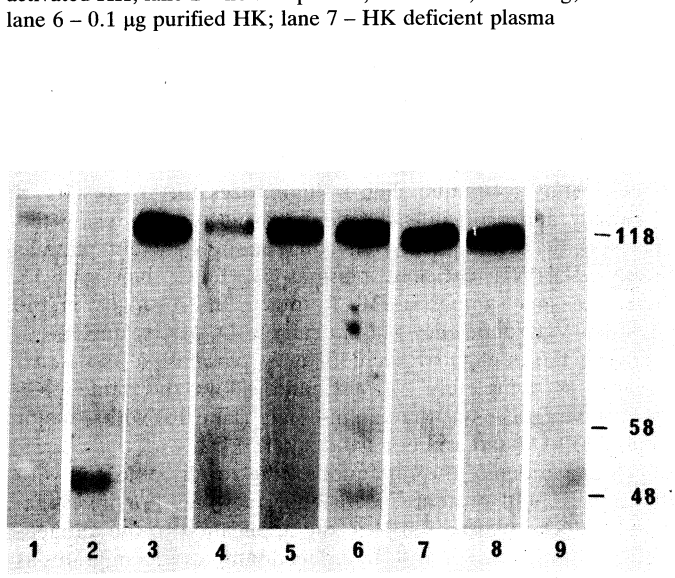

Fig. 6 Immunoblot of reduced protein C and factor IX deficiency plasmas. Aliquots of 40 µl containing 2.5 µl plasma were applied. Lane 1 – 0.1 µg purified HK; lane 2 – kaolin activated HK; lane 3 – normal plasma; lanes 4–6: protein C deficiency plasmas: 4 – a, 5 – c, 6 – d; lanes 7–8: congenital F IX deficiency plasmas: 7 – i, 8 – M; lane 9 – normal human serum

In sample g, another depleted F IX deficiency plasma, H-Kininogen is present primarily in the intact form (Fig. 5, lane 4). In two congenital F IX deficiency plasmas, i and M, H-Kininogen is intact (Fig. 6, lane 7 and 8). All three protein C deficient plasmas contain partially cleaved HK, however, the degree of kininogen cleavage varies and is highest in sample a (Fig. 6, lanes 4–6).

In Fig. 7 the effect of storage of the lyophilized samples for 4 weeks at 37° C on H-Kininogen in one congenital (I, lanes 3 and 4) and one depleted (L, lane 5 and 6) F IX deficiency plasma and one depleted F VIII deficiency plasma (B, lane 7 and 8) can be seen. Storage does not induce changes resulting in different electrophoretic patterns. In the congenitally deficient product, HK is intact and remains intact over the whole incubation period. In the depleted plasma samples, HK has been cleaved already in the starting material. However, further cleavage does not occur during the incubation.

Discussion

The availability of antibodies against most clotting factors allowed the development of immunodepletion procedures for the production of various deficiency plasmas from normal pools. However, during the depletion procedure an intensive contact with foreign surfaces could induce proteolytic processes, which could lead to cleavage, degradation and activation or inactivation of coagulation factors. Such uncontrolled proteolytic processes should be avoided. Therefore, we developed a sensitive technique for the control of previous proteolysis.

In plasma from normal healthy persons, high molecular weight kininogen, a single chain molecule, apparently exsists in two forms, a major one, which migrates on SDS-gels with a MW of 140 kDa, and a minor one, exhibiting a MW of 118 kDa (22). If normal plasma contacts a negatively charged surface such as kaolin, activation of the contact system is initiated and F XIIa is formed (10, 12, 24). This enzyme cleaves and activates prekallikrein and F XI, thereby generating kallikrein and F XIa. Both proteases can activate plasma H-Kininogen, and the activation is associated with the cleavage of the single chain molecule into a two chain form concomitantly with the liberation of bradykinin (16, 17, 25, 26). HK can also be cleaved by a number of other enzymes, such as factor XIIa (27), plasmin (28), trypsin (28) or tryptase, an enzyme, which is liberated from mast cells (29). Factor XIa, trypsin or tryptase reportedly cause a loss of HK-

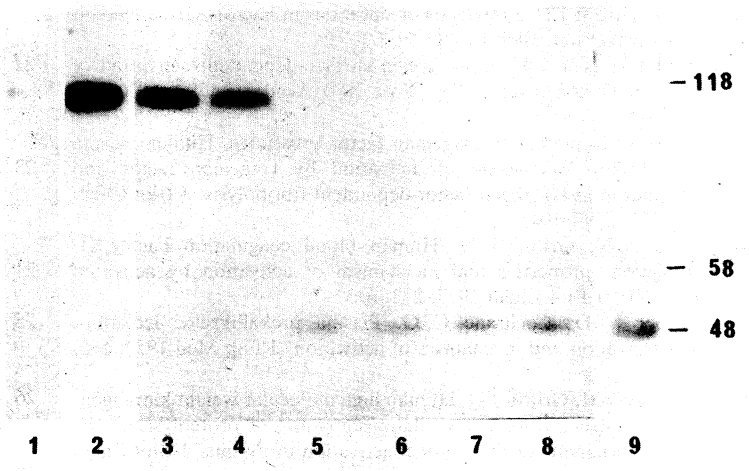

Fig. 7 Immunoblot of reduced plasmas before and after storage at 37° C. Aliquots equivalent to 2.5 µl plasma were applied. Lane 1 – 0.1 µg purified HK; lane 2 – normal plasma; lanes 3 and 4: congenital F IX deficiency plasma sample I: lane 3 before, lane 4 after storage; lanes 5 and 6: depleted F VIII deficiency plasma sample B: lane 5 before, lane 6 after storage; lanes 7 and 8: depleted F IX deficiency plasma sample L: lane 7 before, lane 8 after storage; lane 9 – kaolin activated HK

coagulant activity as well as the generation of HK-fragments different from the ones formed by kallikrein (25, 28, 29).

Since H-Kininogen apparently is very sensitive to proteolysis and can be cleaved by a number of enzymes, it is a good marker to monitor and control the occurrence of proteolytic events.

With our immunoblotting technique we are able to demonstrate the presence of partially or fully cleaved HK in immunodepleted F VIII, F IX or protein C deficient plasma samples of certain companies. In contrast, cleaved HK is not detected in significant amounts in hereditary deficient plasmas nor in steam treated products. These results indicate that proteolytic cleavage of HK is associated with the depletion procedure and must have occurred sometimes during preparation of the artificially depleted plasmas. Interestingly, the degree of HK cleavage in selected products, e.g. factor VIII deficiency plasmas varied from one manufacturer to another. Thus, it appears that the depletion procedures of certain companies induce activation of either the contact coagulation system or another proteolytic pathway, which leads to cleavage of HK.

Interestingly, the HK-fragments, which we can identify in the artificially depleted plasmas correspond to the cleavage products formed by kallikrein. On SDS-polyacrylamide gels we find only bands with MW of 118 kDa and 110 kDa in non reduced samples, and bands with MW of 58 kDa and 48 kDa in reduced samples. Since no other fragments, such as described for HK digested by plasmin or trypsin (28) are present, it appears likely that proteolysis of HK is caused by the factor XIIa-kallikrein pathway.

At present, however, we cannot say whether proteolysis is limited to HK or whether other coagulation factors may also be affected.

It is interesting to note, that H-Kininogen clotting activity in one F VIII and three F IX deficiency plasmas produced by two different companies is very low, namely less than 0.48 units/ml. The presence of sufficient amounts of all other clotting factors except the one to be determined is an important criterium for the use of an immunodepleted substrate plasma in any clotting assay. Thus, the reduced HK activity of 0.39 units/ml observed in sample f, might not be sufficient for a correct determination of F VIII with this substrate.

Whether or not the reduction of HK activity is caused by cleavage of the H-Kininogen molecule cannot be answered conclusively.

Cleavage of H-Kininogen by kallikrein is not associated with a loss of clotting activity (17). However, cleavage by F XIa or tryptase does result in destruction of the clotting activity (25, 29).

Since the HK-fragments, which are detected in the examined plasmas match the kallikrein cleavage products, the reduction of HK clotting activity might not be due to proteolysis, but probably to loss by adsorption to a surface.

In summary, our results indicate that analysis of HK by immunoblotting techniques is a useful test for the occurrence of proteolysis during immunodepletion procedures.

In addition we like to point out that determination of the clotting activity of contact coagulation factors e.g. H-Kininogen, might be a necessary test in the quality control of immunodepleted plasmas.

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