CHROMATOGRAPHIC AND ELECTROPHORETIC SEPARATION OF PLATELET-CLUMPING SUBSTANCE IN RABBIT PLASMA

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The authors previously reported that platelet-clumping substance (SHIMAMOTO and YAMAZAKI, 1958)¹⁾ was detected in heparinized plasma from rabbits administered adrenaline or high molecular weight substance such as agar solution and bacterial endotoxin by an intravenous injection.²⁾ The platelet-clumping substance also came to be observed *in vitro* after weak acidification of plasma in the presence of calcium ions.³⁾ Though its chemical structure is still obscure, the platelet-clumping substance has properties different from those of adenosine-diphosphate (ADP), thrombin, fibrinogen, adrenaline, serotonin, thrombocyte agglutinating factors⁴⁾ or ADP cofactor.⁵⁾ A similar substance was also found in the plasma of patients suffering from acute infectious diseases or thrombosis⁶⁾ and the substance might be related to thrombi-formation in various morbid conditions. So the authors are investigating analysis of the platelet-clumping substance for the genesis of thrombosis and in the present experiments some chemical properties of the platelet-clumping substance of rabbits were studied using column chromatography and electrophoresis.

MATERIALS AND METHODS

I. SAMPLE PREPARATION

Thirty two male rabbits weighing 2.0 to 2.8 kg were used. Twenty one rabbits were bled and the blood was collected for use as a non-treated sample. The blood of the other eleven rabbits received intravenous injections of 0.5 % agar-saline solution (Agar-noble, Nihon Eiken Co., Ltd.) at a dosage of 5 ml/kg was then collected 30 minutes after the injection.

Under light anesthesia (urethane, 1.2 g/kg i.p.), about 80 ml of blood was drained from a carotid artery by way of polyethylene tubing into a siliconized tube containing

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heparin sodium (50 units/ml). The blood was collected for preparation of a platelet-poor plasma. In 10 plasma samples out of the 21 non-treated samples the pH was adjusted to 5.5 (pH meter, model HM 5A, Tōa Denpa Co., Ltd.) with dilute HCl at room temperature. The samples were centrifuged at 1,000 g for 5 minutes to exclude precipitates. Each heparinized plasma collected from the non-treated and the agar-injected rabbits, and the acidified heparinized plasma collected from the non-treated rabbits were subjected to chromatography and electrophoresis at 4°C as follows.

II. COLUMN PREPARATION AND ELUTION SCHEDULE

1. Gel Filtration Column Chromatography

About 20 g of Sephadex G-25, G-50 and G-200 (Pharmacia, Uppsala, Sweden) was used. The mediums used were 0.02 M phosphate buffer, pH 6.6, for G-25 and G-50, and 0.02 M phosphate buffer, pH 7.0, containing 0.5 M NaCl, for G-200. Plasma was dialyzed against the respective buffer for 72 hours, centrifuged at 1,000 g for 15 minutes, and 10 ml of each supernatant was applied to a column (2.5 cm in diameter). The eluate was monitored at 280 m μ by an ultraviolet absorption scanner (Yamakawa Product Co., Ltd., Tokyo, Japan), collected in 15 ml portions by a fraction collector (Tōyō Science Indust. Co., Ltd., Tokyo, Japan) and examined for platelet-clumping activity. The eluate from Sephadex G-200 chromatography was diluted 3-fold with distilled water prior to the test for clumping activity. Meanwhile the absorption curve of each active fraction in the ultraviolet region was measured by a recording spectrophotometer (Shimazu Product. Co., Ltd., Kyoto, Japan).

2. Ion-Exchange Column Chromatography

About 20 g of DEAE-cellulose (0.92 meq/g standard absorbent capacity, Brown, U.S.A.), equilibrated with 0.02 M phosphate buffer, pH 6.0, was used. Plasma was dialyzed and centrifuged to exclude precipitates. Then 10 ml of each supernatant was applied to the column. It was then eluted with the same buffer containing stepwise increments in the NaCl concentration (0.07, 0.4 and 1.5 M). The eluate was monitored for its protein concentration as described before and each eluate (15 ml) examined for platelet-clumping activity.

3. DEAE-Sephadex A-50 Column Chromatography

Dialyzed plasma (10 ml) was chromatographed on a column of about 20 g DEAE-Sephadex A-50 (medium grade, Pharmacia, Uppsala, Sweden) equilibrated with 0.02 M phosphate buffer, pH 6.6, and eluted in a stepwise manner with the same buffer containing 0.07, 0.17 and 0.38 M NaCl. The platelet-clumping activity and the absorption at 280 m μ of the eluates were measured as described above.

The active fractions obtained by the three methods of column chromatography (II. 1, 2 and 3) were separately dialyzed in a cellulose tubing (Visking Co., U.S.A.) against running water for 72 hours and lyophilized for further experiments.

III. ELECTROPHORETIC PROCEDURES

1. Starch-Block Electrophoresis

Starch-block electrophoresis was performed according to the method of KUNKEL and SLATER⁷⁾. Plasma (5 ml) was placed at the starting line of a plastic tray, 26 (L) $\times$ 30 (W) $\times$ 1 (H) cm, filled with hydrolysed starch (Connaught Medical Research Lab., University of Toronto, Toronto, Canada), that had been repeatedly washed with distilled

water and then mixed with veronal buffer, ionic strength 0.05, pH 8.6. Electrophoretic separation of the material was carried out at 4°C with a potential gradient of 5.0 V/cm for 16 hours. Bromphenol blue dye solution was mixed with the sample to serve as an indicator of albumin migration. After electrophoresis the starch block was cut into 1 cm segments beginning at the starting line. Each segment was named 1 cm-fraction (0-1 cm from origin), 2 cm-fraction (1-2 cm from origin) and so on, and placed in a test tube to mix with 1.0 ml of physiological saline. The mixture was agitated 100 times using a bamboo stick. The mixture stood for 30 minutes at 4°C and then the supernatant was examined for platelet-clumping activity. The supernatant was dialyzed against running water for 72 hours and lyophilized for further experiments.

2. *Immunoelectrophoresis*

Immunoelectrophoresis in agar-gel was carried out according to the method of SHEIDEGGER⁸⁾ to identify the plasma component of the active fraction from the starch block. Goat antiserum to rabbit serum (Hyland Lab., Los Angeles, Calif., U.S.A.) was used as the antibody.

3. *Paper Electrophoresis*

Cellulose acetate membrane (Separax, Jōkō Indust. Co., Ltd., Tokyo, Japan) electrophoresis was made on paper electrophoresis equipment (Jōkō Indust. Co., Ltd., Tokyo, Japan) according to the method described by ARONSSON and GRÖNWALL⁹⁾. Veronal buffer, pH 8.6, $\mu=0.06$, was generally employed. Ponceau 3R, nigrosin B, alcian blue and toluidine blue were used for staining.

IV. ASSAY FOR PLATELET-CLUMPING ACTIVITY

The method was essentially the same as reported previously^{2,3)}. Blood was collected from healthy male rabbits weighing 2.2 to 2.9 kg. Disodium ethylenediamine tetraacetate (EDTA) (final concentration: 0.1 %) was used as an anticoagulant. The blood was centrifuged at 100 g for 30 minutes at 4°C. The resulting supernatant (platelet-rich plasma) was then centrifuged at 1,000 g for 15 minutes at 4°C and platelets in the sediment layer were washed twice with physiological saline and then resuspended in saline of one half of the volume of the original blood sample. The pH of the saline was adjusted to 7.4-7.6 with a dilute NaHCO_3 solution. The washed platelet suspension (7×10^5 - $10 \times 10^5/\text{mm}^3$) showed no spontaneous clumping under microscopic examination. Meanwhile platelet-rich plasma was collected using sodium citrate (final concentration: 0.38 %).

One drop of the sample was mixed one drop of the normal platelet-saline suspension in a paraffin cup by gently sucking and blowing 10 times with a siliconized Pasteur-pipette at room temperature. The mixture was immediately transferred to a siliconized slide, covered gently with a siliconized cover-slip and observed for the occurrence of platelet clumping under a regular light microscope ($\times 450$). The assay for platelet clumping was carried out 3 times and within 6 hours after collecting platelets from different rabbits. The plasma which induced clumping of platelets to the positive or marked positive degree was found to contain the platelet-clumping substance. In some cases the platelet-clumping activity in citrated platelet-rich plasma was examined. The titer of platelet-clumping activity in the plasma and eluate was determined by serial dilution.

RESULTS

I. PLATELET-CLUMPING ACTIVITY IN PLASMA SAMPLES

Platelet-clumping activity was found in 3 of 11 plasma samples from the nontreated rabbits. It was found in 10 of 11 plasma samples from agar-injected rabbits and in all 10 acidified plasma samples from non-treated rabbits. The degree of activity was almost the same with washed platelets in platelet-saline suspension as with unwashed in citrated platelet-rich plasma. The platelet-clumping activity was found even after 8-16 fold dilution of the plasma with physiological saline.

The 10 active plasma samples from agar-injected rabbits (agar-plasma), and all 10 acid-activated plasma samples were used to separate the platelet-clumping substance. Eight of 11 plasma samples from non-treated rabbits, with a negative platelet-clumping activity were used as control samples (control plasma).

II. COLUMN CHROMATOGRAPHY

1. *Gel Filtration Column Chromatography*

The typical elution curves from Sephadex G-25, G-50 and G-200 columns, representing the protein fraction and the platelet-clumping activity of the plasma preparation, are shown in FIG. 1. Using these columns, the platelet-clumping substance was not separated from the main protein peaks. About 90 % of the protein that was applied to the column was recovered. About 90 % of the platelet-clumping activity was recovered from G-25 and G-50, and about 80 % from G-200 column chromatography experiments. The platelet-clumping substance was not retained, but rather passed through the G-25 and G-50 columns. In G-200 column chromatography the platelet-clumping substance was detected at the end of the third protein peak, which was identified as an albumin fraction by paper electrophoresis. Similar results were found using 5 agar-plasma samples and 8 acid-activated plasma samples. On the other hand, the effluents after gel filtration column chromatography of all 5 control plasma samples failed to show clumping activity in any of the fractions.

2. *Ion Exchange Column Chromatography*

The typical chromatographic curve of the proteins from a DEAE-cellulose column and the distribution of the plasma platelet-clumping activity are shown in FIG. 1. In this column chromatography, the platelet-clumping substance was separated from the main protein peaks. About 90 % of the protein was recovered, while 10 % of the platelet-clumping activity was recovered. The platelet-clumping substance was eluted with 1.5 M NaCl containing phosphate

FIG. 1. Column chromatography of rabbit plasma. Plasma samples were obtained from rabbits injected with 0.5% agar-solution (5 ml/kg i.v.) before the collection. The elution curves of the protein fractions are represented by optical density at 280 m μ . The platelet-clumping activity of the eluates is expressed by + (positive) or - (negative).

buffer, pH 6.0, in the cases of 2 agar-plasma samples and 2 acid-activated plasma samples. The 2 control plasma samples did not show the clumping activity in any of the chromatographic fractions.

3. DEAE-Sephadex A-50 Column Chromatography

The typical chromatographic curve from a DEAE-Sephadex A-50 column

is shown in FIG. 1. In this column chromatography, the platelet-clumping substance was separated from the main protein peaks. The recovery values for protein and platelet-clumping activity were about 90 % and 10 % respectively. The platelet-clumping substance, after eluted with 0.17 M NaCl-0.02 M phosphate buffer, pH 6.6, appeared after the third protein peak. Analysis by paper electrophoresis showed that the first peak of protein is α and β -globulin, the second peak is haptoglobulin, the third peak albumin; and the last peak γ -globulin. The platelet-clumping activity was usually detected just after the albumin peak in the cases of 3 agar-plasma samples and 2 acid-activated plasma samples. Two control plasma samples did not show the clumping activity in any of the fractions after DEAE-Sephadex A-50 column chromatography.

III. ELECTROPHORESIS

1. *Starch-Block Electrophoresis*

Starch-block electrophoresis was performed on 10 plasma samples from agar-injected rabbits (agar-plasma). After electrophoresis albumin-bound bromphenol-blue was detected in most cases between the 8 cm- and 9 cm-fraction. The most darkly stained fraction was 8.3 ± 0.26 cm-fraction (average and standard error in the 10 cases). The platelet-clumping activity was found in the eluates of the fractions between 8 cm and 11 cm. The fraction which showed the strongest platelet-clumping activity was the 9.6 ± 0.31 cm-fraction in average of the 10 cases. In 2 of the 10 cases, the platelet-clumping activity was detected in a fraction containing a component of faster mobility than that of albumin and was not detectable in the main albumin fractions. In the other 8 cases the platelet-clumping activity was contaminated by the albumin fractions.

The albumin-bound bromphenol-blue was detected between the 8 cm- and 10 cm-fractions and the platelet-clumping activity was found in the eluates of the fractions between 9 cm and 11 cm after starch-block electrophoresis of 3 acid-activated plasma samples. In 1 of the 3 cases, the platelet-clumping activity was detected in a fraction containing a component of faster mobility than that of albumin but was not found in the albumin fractions.

Starch-block electrophoresis was also performed on 8 control plasma samples. Albumin-bound bromphenol-blue was detected between the 8 cm- and 9 cm-fractions. The most darkly stained fraction was at 8.6 ± 0.07 cm in average of the 8 cases. The platelet-clumping activity was also found in the eluates of the fractions between 9 cm and 11 cm, although the plasma did not show any clumping activity before electrophoresis. The strongest platelet-clumping activity was found in the 9.6 ± 0.14 cm-fraction in average. In 3 of the 8 cases the platelet-clumping activity was found in a fraction with a faster mobility than that of albumin. In the case of the 3 plasma samples

from non-treated rabbits which showed platelet-clumping activity, albumin was found in the 9.0 ± 0.00 cm-fraction and the platelet-clumping activity in the 10.3 ± 0.31 cm-fraction in average.

In any of these plasma samples, an anticoagulation activity, which originated from added heparin, was found in the fractions with faster mobility than that of the platelet-clumping substance. It was found usually in the eluates of the fractions between 15 cm and 18 cm (average: 16.9 ± 0.21 cm). The typical starch-block electrophoretic pattern for the albumin-bound bromphenol-blue and for the platelet-clumping activity and the anticoagulation activity in plasma is shown in FIG. 2.

FIG. 2. Starch block electrophoresis of rabbit plasma. Bromphenol blue-stained albumin zone is shown as shadowed area; the platelet-clumping activity and the anticoagulation activity are shown as + or -. 0 means starting line.

2. Immuno-electrophoresis

To clarify some possible relationship between plasma proteins, especially albumin and the platelet-clumping substance, immuno-electrophoresis was performed on the eluates from starch-block electrophoretic fractions of 2 control plasma samples and 4 agar-plasma samples. The typical immuno-electrophoretic patterns for the albumin-precipitation line and for the platelet-clumping activity of the eluate are shown in FIG. 3. The eluate of the 7 cm-fraction showed a marked precipitation line against albumin but not against platelet-clumping activity. The eluates of the 8 cm- and 9 cm-fractions with their marked platelet-clumping activity exhibited a moderately dense precipitation line against albumin. The eluate of the 10 cm-fraction with platelet-clumping activity gave no precipitation line. In all the 6 cases similar results were obtained and the clumping activity was found in one or more fractions containing albumin and/or component of faster mobility than that of albumin (pre-albumin fraction).

FIG. 3. Immunoelectrophoresis on the eluates from starch-block electrophoretic fractions of rabbit plasma. Goat antiserum to rabbit serum was used as the antibody. The numbers of the left side indicate the fractions from which the eluate was collected. In the right side, the grades of platelet-clumping activity are shown. The bottom section shows the electrophoretic pattern of rabbit plasma as a control. White precipitation lines against albumin are demonstrated with the fractions of 9, 8 and 7, indicating the difference between the albumin-precipitation line and the clumping activity.

IV. SOME PROPERTIES OF THE PLATELET-CLUMPING SUBSTANCE

The active preparations isolated from plasma by starch-block electrophoresis, DEAE-cellulose or DEAE-Sephadex A-50 column chromatography, were dialyzed against running water for 72 hours. These eluates induced platelet-clumping not only in platelet-saline suspension but also in citrated platelet-rich plasma even after the dialysis. The platelet-clumping activity was neither destroyed by heating (100°C, 15 minutes) nor interfered with by the addition of 1% EDTA in final concentration. These failed to demonstrate any distinct peak around 260 m μ .

DISCUSSION

Though the chemical structure of the platelet-clumping substance is still obscure, the present experiments indicate that the eluates from DEAE-cellulose or DEAE-Sephadex A-50 column chromatography and starch-block electrophoresis have the ability to clump platelets in a manner similar to that found

in plasma collected from agar-injected rabbits²⁾ and the acid-activated plasma³⁾. It was not dialyzable. These results suggest that the active platelet-clumping substance may be of high molecular weight and acidic in nature.

After electrophoresis of plasma, coagulation factors I, II, V, VII, VIII, IX, X, XI, XII and XIII were detected in globulin fractions¹⁰⁾. BRINKHOUS *et al.*^{4,11)} reported that thrombocyte agglutinating factors are detectable in crude globulin fractions and destroyed by heating for 10 minutes at 56°C. ADP-cofactor, reported by BORN *et al.*⁵⁾, is present in euglobulin fractions and is not heat stable. Plasma co-factor, reported by DEYKIN *et al.*¹²⁾, is present in the crude globulin fractions. These substances require calcium or magnesium ions, or ADP in order to clump washed platelets. Recently BARNHART *et al.*¹³⁾ reported that a production of fibrinogen degradation (FSP) actively aggregated platelets and NIEWIAROWSKI¹⁴⁾ showed an early fibrinogen degraded product (FDP) to aggregate platelets. These experiments were carried out using platelet-rich plasma. The platelet-clumping substance observed by the authors clumped washed platelets even with 1% EDTA added and was separated from the anticoagulation activity by the starch-block electrophoresis. It was detected in the pre-albumin fraction by starch-block electrophoresis and did not show any precipitation line against plasma proteins in the immunoelectrophoretic findings. The platelet-clumping substance present in plasma was lost by heating for 30 minutes at 56°C³⁾, but once it was isolated from the plasma proteins by column chromatography or electrophoresis, its activity was not destroyed by heating. It showed no peak at 260 m μ . It is concluded, therefore, that a clear difference exists between the substance observed by the authors and ADP or the other plasma factors reported.

In the present experiments the platelet-clumping activity was found in electrophoretic samples from albumin or pre-albumin fractions even when non-treated rabbit plasma which showed no platelet-clumping activity was applied. The starch-block electrophoresis may activate the platelet-clumping substance or remove some inhibitor in the plasma as in the case of acidification³⁾.

In 1961, GAARDER and his associates¹⁵⁾ found the aggregation of platelets induced by ADP. For the last few years a number of studies have developed concerning the effect of ADP and the other low molecular weight compounds on platelets. However, the platelet-clumping substance described by the authors in 1958¹⁾, differed from these described products. In the present experiment, the platelet-clumping substance was separated from plasma proteins and its chemical property might be of high molecular and acidic in nature. It is very interesting that abundant acid mucopolysaccharide consists in surface coat of platelets¹⁶⁾. It may be related to clumping of platelets. Recently DAVEY and ESNOUR¹⁷⁾ separated a substance from the venom of the Okinawan pit viper (*Trimeresurus okinavensis*) that causes the aggregation

of human platelet. The purified venom fraction is a protein-polysaccharide. The structure of the substance observed by the authors may be partially similar to the above mentioned substance, however, the platelet-clumping substance was found in plasma collected from the rabbits injected with high molecular weight substance or adrenaline²⁾ and from humans with thrombosis, infection or tissue destruction⁶⁾. It was also found in plasma after the weak acidification which could be observed in ischemic or inflammatory tissues locally⁸⁾. These results may suggest that a precursor of the platelet-clumping substance is present in plasma and activated by inflammatory or ischemic responses. The result obtained from the present experiment implies the importance of further research, suggesting the existence of a phylogenetically old mechanism¹⁸⁾ which counteracts the bleeding regardless of fibrinogen fibrin system.

SUMMARY

The heparinized plasma collected from 11 non-treated, 10 agar-injected rabbits and 10 heparinized acidified plasma samples were eluted by column chromatography (G-25, G-50, G-200, DEAE-cellulose and DEAE-Sephadex A-50) or treated by starch-block electrophoresis. Using column chromatography for G-25, G-50 or G-200, the platelet-clumping activity was found in the protein fractions. Using chromatography of DEAE-cellulose, the platelet-clumping activity was eluted in 1.5 M NaCl containing phosphate buffer, pH 6.0. Using chromatography of DEAE-Sephadex A-50, it was eluted in 0.17 M NaCl containing phosphate buffer following the albumin fraction. Using starch-block electrophoresis, the platelet-clumping activity was found in the albumin band and/or in fractions containing a component of faster mobility than that of albumin (pre-albumin fraction). Platelet-clumping activity was also detected in electrophoretic extracts of plasma from non-treated rabbits which, prior to electrophoresis, had not shown any platelet-clumping activity. Starch-block electrophoresis may serve to activate the platelet-clumping substance or remove some inhibitor in the plasma. The eluates collected through the above mentioned procedures clumped platelets not only in citrated platelet-rich plasma but also in platelet-saline suspension without calcium ions. It was not dialyzable against running water for 72 hours; it was heat-stable at 100°C for 15 min.; it failed to demonstrate a peak around 260 m μ in the ultraviolet absorption test.

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