Intravascular Hemagglutination Studied
with the Living Extracorporeal Eye

By Arthur J. Seaman, C. Lawrence Lutcher and Charlotte Moffat

This paper is an account of our direct and filmed observations of intravascular events during perfusion of the microcirculation with admixtures of incompatible bloods. The events were visualized in our previously described experimental preparation, The Living Extracorporeal Eye. Additionally, serial assessments of the coagulation activities in arterial and venous blood samples and histopathologic studies of the vessels and their contents by light and electron microscopy during such incompatible blood perfusions are presented. The influence of a variety of procedures and therapeutic agents in altering these events has been investigated and is described.

Methods

The Living Extracorporeal Eye has been described in detail elsewhere and evidence of viability and metabolic activity of its tissues is presented there. Briefly, bovine eyes were removed immediately after slaughter. The ciliary artery was catheterized and the microcirculation was cleared first with Kreb's solution (less Ca\(^2\) ions), then with oxygenated silicone-handled anticoagulated bovine blood. Extracorporeal perfusion with bovine blood was continued for a period of 20 to 30 minutes, then thorough flushing of this blood from the circulation with Kreb's solution (less calcium ions) was carried out until the venous return was free of erythrocytes on direct microscopic observation.

The eye was suspended in a special receptacle. If visualization was by incident light, observations were made by focusing on the retina through the pupil with a Zeiss ophthalmologic operating microscope with a coaxial light source. When the retinal vessels were studied in transillumination, a scleral-choroidal window was made and light directed by mirror through the pupil to light the window. In the latter instance, observations were made through a laboratory microscope focused on the retinal vessels exposed in the window. With both types of illumination, cinephotography was carried out through the appropriate microscopes using a Bolex 16 mm. motion picture camera. Kodachrome IIA photoflood film was used.

To determine the alterations in coagulation components induced by intravascular hemagglutination, eyes were next perfused with 30 ml. of human blood of a single type. In half of the eyes this single blood type was continued.

In half of the eyes, after perfusion with 30 ml. of a single blood type, the 20 ml. syringe driven by the perfusion pump, was filled with two 10 ml. aliquots of incompatible bloods and mixed. The venous outflow was collected from first 10 ml. of mixed in
compatible blood perfused. That remaining in the syringe constituted the arterial specimen. Similar collections were made when the same blood type was perfused alone.

Determinations were made of platelets, fibrinogen, prothrombin time, partial thromboplastin time, P & P activity, and hematocrit from arterial and venous samples.

For interspecies hemagglutination the eye was perfused in pulsatile flow with native bovine arterial blood via a silastic catheter inserted percutaneously into the subcutaneously translocated carotid artery of a donor cow. Via a siliconized Y tube, diluted human blood was admixed with the native bovine blood at the entrance to the ciliary artery catheter. Heparin (200 u/ml final dilution) and heparin (200 u/ml final dilution) plus 1 per cent edetic acid (EDTA) were the diluents constituting half of the final volume of the added human blood.

To study the effect of osmotic hemolysis, equal volumes of citrated human blood and distilled water were admixed in the common connecting tubing entering the ciliary artery catheter by simultaneously driving separate syringes with the same Sage pump.

Paraffin sections were cut with a Porter-Blum microtome from retinal tissue, fixed in Zenker's solution, and stained with periodic acid Schiff hematoxylin. Tissues for electron microscopic sectioning were fixed with osmium tetroxide, embedded in Swiss araldite, cut on a Leitz ultratome, and examined and photographed with an RCA EMU 3F electron microscope.

Immediately prior to use, human bloods of differing types were collected via veno-
Puncture in siliconized syringes and added to one-tenth volume of one per cent EDTA in saline or one-tenth volume of 3.8 per cent trisodium citrate solution in siliconized flasks. The blood was overlaid with oxygen and swirled gently until oxygenated. Bloods with low P & P values were drawn from donors receiving long-term therapy with oral indirect anticoagulant drugs.

In one series of experiments, anti-A antibodies (Dade reagent serum) 0.1 ml. per 1.0 ml. of Type A human blood was perfused instead of two incompatible bloods.

By differential centrifugation the plasma supernatant of blood spun at 500 r.p.m. for 10 minutes, was respun at 15,000 r.p.m. for 10 minutes and the supernatant plasma of the latter recombined with saline washed autologous erythrocytes in the original hematocrit to produce a leukocyte and platelet poor specimen. Such preparations from two incompatible bloods were simultaneously infused.

In another set of experiments, platelet poor plasmas from incompatible bloods were separately incubated at 56 C. for 15 minutes and then spun at 2,500 r.p.m. for 15 minutes to remove the heat precipitated protein. The supernatant of each was combined with its autologous erythrocytes in the original hematocrit and the reconstituted defibrinated incompatible blood pairs were simultaneously perfused.

Streptokinase (Kinolysin—Merck, Sharp, and Dohme), 12,500 units per 1 ml. blood or streptokinase-streptodornase (Varidase—Lederle), 20,000 units per ml. of plasma or
Fig. 3.—Incompatible blood transfusion reaction showing a “saddle embolus” of aggregated erythrocytes and fibrin at the impact wall of an arterial “Y” branching. (See arrow)

Urokinase (Abbott), 620 units per ml. plasma or 5M urea solution were perfused when there was well marked fibrin deposition in the veins. Other preparations used were:
- Hydrocortisone (Solu-Cortef, Upjohn), 50 mg. per ml.
- Heparin (Upjohn), 1,000/units/ml.
- Clinical Dextran (Cutter) 6 per cent solution av. M. W.-70,000
- Benadryl Hydrochloride (Park-Davis and Co.), 10 mg. per ml.

RESULTS

A smooth red uniform arterial flow was apparent and continued throughout perfusion of the eye with any single type of human blood. In this sense all blood types used were “compatible.” Uninterrupted flow proceeded into the smaller arterioles, capillaries, and returned through venules and veins. After perfusion with one type of blood was established, a second blood of a type incompatible with the first was added to the arterial inflow via a three-way stopcock and a model No. 234-4 Sage pump driven siliconized syringe.

Immediately thereafter, the arterial flow became granular and then grossly aggregated into a moving series of long red dashes separated by bright
liquid spacings largely free of cellular elements (Fig. 2). At Y-shaped branchings of the artery it became apparent that large red cell aggregates were frequently linked by tenuous fibrous strands spanning the plasma gaps into chains of as long as five to six aggregates. When the initial aggregate entered one side of the “Y” it dragged the entire chain behind it up that channel resembling a series of railway boxcars following a locomotive onto a siding. Occasionally a short chain would become impacted at the “Y” like a saddle embolus (Fig. 3). Additional aggregates sluggishly rolled over its sticky surface entering one or the other side channel. The liquid in the bright interspacings flowed into both arms of the “Y.” As the large red cell aggregates moved into progressively smaller arterioles, the lumen finally became plugged and the play of the flow in the capillary fields ceased distal to this impaction. The blood in the venules draining these stilled capillaries became static. Where stilled venules entered the collecting veins slightly refractile brick-red deltas marked each site of junction. Flow in the collecting veins continued from upstream. Gradually the deltas grew by a combination of slow extrusion plus accretion on their downstream sides until they

Fig. 4.—A many-tailed partial cast of the venous tree resulting from incompatible blood transfusion reaction.
joined one another in a long continuous strand. Similar strands formed in other venules and these fused together with progressive thickening where the collecting veins joined. Finally a many-tailed partial cast of the venous tree was formed (Fig. 4). Usually this process developed over a period of 20 to 40 minutes. Flow-by continued lateral to the forming venous cast. Similar phenomena occurred when Type A blood and anti-A antibodies were perfused simultaneously.

Sections of such a venous many-tailed case are shown in Figures 5 and 6 as viewed by light microscopy and electron microscopy, respectively, and indicate a central core of aggregated erythrocytes surrounded by fibrin and aggregated platelets.

In our studies of the differences in coagulation components between arterial and venous specimens we found a modest decrease in platelets and mild lengthening of the partial thromboplastin time. This was not different when a single blood type was perfused or when incompatible pairs of bloods were mixed and infused. (See Table 1). Since hematocrits were unchanged, this decrease in platelet number may represent a utilization of platelets for maintaining vascular integrity although adhesion to collagen on the exterior of the eyeball has not been excluded.

Disaggregation of the obstructing arterial red cell masses and lysis of venous many-tailed cast strands followed perfusion with streptokinase dissolved in human plasma, or with urokinase (Fig. 7 and 8), or with 5M urea solution. Dissolution did not occur when perfusion was with Kreb's solution or clinical dextran solution. Addition of hydrocortisone (3mg./1 ml. blood) or benadryl (3mg./1 ml. blood) to each of the reacting bloods prior
**Table 1.—Quantification of Blood Components Before and After Perfusion of the Living Extracorporeal Eye with Blood of a Single Type or with a Mixture of Two Incompatible Eyes**

<table>
<thead>
<tr>
<th></th>
<th>Pre Single Blood Type</th>
<th>Post</th>
<th>Pre Two Incompatible Bloods</th>
<th>Post Admixed Bloods</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platelets</strong></td>
<td>238,000/mm.$^3$</td>
<td>173,000/mm.$^3$</td>
<td>242,000/mm.$^3$</td>
<td>169,000/mm.$^3$</td>
</tr>
<tr>
<td><strong>Fibrinogen</strong></td>
<td>225 mg.%</td>
<td>229 mg.%</td>
<td>225 mg.%</td>
<td>224 mg.%</td>
</tr>
<tr>
<td><strong>P T T</strong></td>
<td>59.9 sec.</td>
<td>66.3 sec.</td>
<td>59.8 sec.</td>
<td>67.4 sec.</td>
</tr>
<tr>
<td><strong>Quick</strong></td>
<td>13.9 sec.</td>
<td>14.0 sec.</td>
<td>13.6 sec.</td>
<td>13.8 sec.</td>
</tr>
<tr>
<td><strong>HCT</strong></td>
<td>41.0%</td>
<td>41.9%</td>
<td>41.2%</td>
<td>41.0%</td>
</tr>
</tbody>
</table>

Paired coagulation studies.
Mean of 24 determinations. See text.

**Fig. 6.—Electron microscopic view of venous cast. Agglutinated erythrocytes, some showing lysis with fibrin deposition on surface. Intact platelets are seen in same field.**

to mixing or of 6 per cent clinical dextran (0.2 ml. per 1 ml. blood) did not alter the sequence of events described above.

Reduction of leukocyte value of 2585/cu. mm. and of platelets to 68,750/cu. mm. by differential centrifugation did not prevent or alter the aggregation of erythrocytes or the deposition of fibrin resulting from reaction of Type A human blood with anti-A antibodies. Addition of heparin (125 U./ml.
Fig. 7.—Venous red cell and fibrin cast before fibrinolytic therapy with urokinase.

blood) to the EDTA anticoagulated reacting bloods, however, reduced the degree of arterial aggregation to a granularity of flow (Fig. 9). Capillary perfusion remained vigorous and venous erythrothrombi were completely prevented. Perfusion with incompatible pairs of human bloods altered only by heat-defibrination of their EDTA anticoagulated plasmas, resulted in loose arterial erythrocyte aggregates which were easily disassociated to permit passage through capillaries. Venous cast formation did not occur. Admixtures of EDTA anticoagulated incompatible bloods from donors receiving long-term indirect anticoagulant therapy in doses that reduced P & P values (Owren and Aas) to 22 per cent and 15 per cent, respectively, showed as marked arterial aggregation of erythrocytes and venous cast formation as did bloods with normal P & P values.

Native bovine blood delivered in pulsatile flow through a percutaneously placed intra-arterial catheter from a donor cow perfused the microcirculation of The Living Extracorporeal Eye smoothly for periods as long as several hours. Addition of diluted human blood with heparin (200 U./ml. final concentration) resulted in prompt massive aggregate formation which promptly occluded the microcirculation. Addition of diluted human blood plus
Fig. 8.—Same field as in Figure 6 showing lysis during urokinase infusion. There was subsequent complete clearing of the lumen.

the same final concentration (200 U./ml.) of heparin plus edetic acid 1 per cent final concentration) markedly reduced this interspecies hemagglutination. When both calcium ion binding and heparin were present, microcirculatory flow continued though at a reduced rate. No erythrothrombosis developed. Heparin alone was not able to accomplish the same effect in the doses utilized.

When osmotically lysed red cells of a single human blood type were perfused, erythrothrombosis developed unless flow rates were extremely high. Slower rates that simulated stasis or stagnation secondary to minute long interruption of flow by stopping the pump led to erythrothrombotic cast formation.

**DISCUSSION**

Naunyn and Landois are cited by Krevans et al., as recognizing as early as 1873 and 1875, respectively, that injection of hemolysate or heterologous red blood cells into dogs led to in vivo depletion of prothrombin, platelets, and fibrinogen in individuals suffering blood transfusion reactions.
Fig. 9.—Marked modification of blood transfusion reaction with addition of heparin (125 units/ml).

Our experimental model differs in a number of aspects from the living human patient. Blood passing through the microcirculation is not monitored by an active reticuloendothelial structure other than the endothelial cell itself and makes but a single pass through the microcirculation. In the experiments reported above, the silicone handled blood specimens are each mixed with one-tenth volume of 3.8 per cent trisodium citrate solution or of 1 per cent EDTA in saline. These anticoagulants should bind calcium ions sufficiently in this concentration to inhibit intravascular clotting.

We found, however, that venous erythrothrombi did form multicentrically and join by accretion until many-tailed partial casts in the venous tree were apparent. These still permitted blood to flow by. Each brick red delta from which these unique venous clots were anchored seemed to form in the mouth of a postcapillary venule at its junction with the next larger venous channel draining it. Usually these postcapillary venules had been stilled as aggregated erythrocyte masses obstructed the precapillary arterioles feeding the interposed capillary bed. We reason that hemagglutination per se did not cause the intravascular coagulation. Attempts by us to demonstrate de-
pletion of coagulation factors by admixture of incompatible bloods in vitro have been unrewarding. Alteration of the endothelial cells secondary to impaired perfusion with oxygenated blood and the venous stasis resulting when the aggregated erythrocyte masses obstructed precapillary arterioles may combine to trigger multiple loci of clotting whose central cores of aggregated erythrocytes have fibrin and platelet deposition binding the surface. These latter deposits are thought from the data of Table 1 to have resulted from localized or surface thrombin evolution. The hemagglutination facilitates stasis. The loss of integrity of the red cell membrane may provide the stimulus for such surface coagulation. Osmotically lysed erythrocytes cause similar erythrothrombi when stasis is induced. If flow rate remains high, however, the red cell coagulum is flushed along by the stream before fixation to the endothelium can be achieved. We believe that altered oxygenation of the endothelial cells may contribute to this binding of the erythrothrombosis at multiple sites in venules though it is apparent that deltas grow by accretion in flowing streams as well. Heparin alone seems incapable of blocking interspecies hemagglutination and erythrothrombosis. The red cell stroma thus seems to act as a tissue thromboplastin and possibly also as a source of calcium ion when exposed by hemolysis be it secondary to hemagglutination or osmotic hypotonicity. The reaction seems largely to involve erythrocyte and static endothelial cell surfaces. Autocatalytic coagulation with general depletion of plasma coagulation factors and platelets does not seem to occur as judged from the failure of fibrinogen values and prothrombin times to alter in draining venous blood as compared with arterial blood.

**Summary**

Direct and filmed observations of events during intravascular hemagglutination have been made using our Living Extracorporeal Eye preparation. Agglutinated erythrocyte masses in the arterioles are separated by plasma gaps but tied to one another in short chains by fibrin threads and have a surface deposition of fibrin. This fibrin forms under circumstances that make autocatalytic coagulation unlikely. This seems to indicate that if thrombin evolution occurs, it must be localized to the areas of exposed erythrocyte stroma and adjacent endothelial cell surfaces altered by deoxygenation.

These masses plug some of the microcirculatory channels and venous many-tailed casts build from multicentric sites at the exits of venules with stillled flow. The light and electron microscopic structure of such casts shows a core of aggregated erythrocytes with surface fibrin and platelet deposition.

The above events are not prevented by prothrombinopenic, leukopenic or thrombocytopenic reacting bloods but are abolished by resuspending from each of the reacting bloods, the autologous erythrocytes, leukocytes, and platelets in the supernate of its centrifuged heat-defibrinated plasma.

Hydrocortisone, benadryl and 6 per cent clinical dextran do not alter the reaction.

When calcium ion binding agents are also present, heparin abolishes the fibrin formation and sharply reduces the magnitude of erythrocyte aggregates
so that vigorous perfusion of the microcirculation continues. Heparin alone is ineffective in suppressing interspecies hemagglutination and erythothrombus formation when non-anticoagulated bovine blood is intermixed with heparinized diluted human blood.

The obstructing arteriolar masses and the many-tailed venous cast induced by hemagglutination, are promptly and completely disaggregated by plasma plus streptokinase or by urokinase or by 5M urea solution perfusion.

Osmotically lysed erythrocytes lead to similar erythrothrombus only when stasis is induced by interrupting arterial perfusion for a minute or more.

**SUMMARIO IN INTERLINGUA**

Observationes directe e cinematographic del evenimentos occurrente durante hemagglutination intravascular esseva effectuate per medio de nostre Vive Oculo Extracorporee.

Massas agglutinate de erythrocytos in le arteriolas es separate per lacunas de plasma sed interligate in curte catenas per filos de fibrina e, in plus, ha depositos superficial de fibrina. Iste fibrina se forma sub circumstantias que rendre improbabile un mechanismo de coagulation autocatalytic. Isto pare indicar que si thrombina es evolutive, le evolution de illo debe esser localisate in le areas de exponite stroma erythrocytic e de adjacent superficies de cellulass endothelial que es alterate per deoxygenation.

Iste massas obstrue certes del canales microcirculatori, e venose cylindros multicaudate es formate in sitos multicentric in le exitos de venulas con fluxo arrestate. Le structura de tal cylindros include, secundo studios a microscopia optic e electronic, un centro de aggregete erythrocytos con fibrina superficial e depositos de plachettas.

Le supra-descritbe evenimentos non es prevenite per sanguine a reaction prothrombinopenic, leucopenic, o thrombocytopenic, sed illos es abolite quando in le caso de cataun del sanguines reagente, le autologe erythrocytos, leucocytes, e plachettas es resuspendite in le fluido supernatante de su centrifugate thermo-defibrinate plasma.

Le reaction non es alterate per hydrocortisona, benadril, o un solution de 6 pro cento de dextrano clinic.

Quando agentes a ligation de iones de calcium es etiam presente, heparina aboli le formation de fibrina e reduce marcartamente le magnitude del aggregatos de erythrocytos de maniera que un vigorose perfusion del microcirculation continua. Heparina sol es inefficace in supprimer interspecific hemagglutination e formation de erythrothrombos quando non-anticoagulate sanguine bovin es intermiscite con heparinisate diluite sanguine human.

Le obstruente massas arteriolar e le multicaudate cylindros venose que es inducite per hemagglutination es disaggregate prompte- e completemente per perfusion con plasma plus streptokinase o de urokinase o de un solution de 5 M urea.

Osmoticamente lysate erythrocytos resulta in un simile erythrombose solo quando state es inducite per le interruption del perfusion arterial durante periodos de un minuta o plus.

**ACKNOWLEDGMENT**

We wish to acknowledge the Pacific Meat Company’s cooperation in allowing us to obtain bovine eyes.

**REFERENCES**


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ARTHUR J. SEAMAN, C. LAWRENCE LUTCHER and CHARLOTTE MOFFAT

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