SPONTANEOUS EXTRAVASATION OF ERYTHROCYTES IN THROMBOCYTOPENIA

SPONTANEOUS EXTRAVASATION OF ERYTHROCYTES IN EXPERIMENTAL THROMBOCYTOPENIA: A STUDY OF THE ERYTHROCYTE CONTENT OF THORACIC DUCT LYMPH IN NORMAL AND IN THROMBOCYTOPENIC ANIMALS

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BY

GREGORIOS PAZIONIS, M. SC., M. ENG.

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TO MY WIFE

THERESA IRENE NOWAK

for her compassion

patience and understanding

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AUTHOR: Gregorios Pazionis, Ptyhion (Aristotelian University, Greece)

M. Sc. (Dalhousie University)

M. Eng. (McMaster University)

SUPERVISOR: Dr. J. Hirsh, Professor of Pathology

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ABSTRACT

A. A comprehensive and in depth review of the increased permeability of the vascular wall in thrombocytopenia was expedited. As part of this review, the following areas were covered: i. irradiation induced thrombocytopenia and the vascular wall defect; ii. anti-platelet serum induced thrombocytopenia and the vascular wall defect; iii. the endothelium and current theories on the role of platelets in supporting endothelial cell homeostasis; iv. the direct effect of irradiation and of anti-platelet sera on endothelium cell function and vitability; v. current models of explaining the endothelial cell changes and the escape of erythrocytes from the macroscopically intact capillaries and venules in thrombocytopenia; vi. a comparative analysis of the two thrombocytopenia models.

B. Although both qualitative and quantitative studies of the spontaneous extravasation of erythrocytes in irradiation induced thrombocytopenia have been made in the past, only qualitative electron microscopic studies have been reported up until now for the anti-platelet serum thrombocytopenic model. In the present work, we established such a quantitative estimate by studying the

changes of the cellular content of the thoracic duct lymph in anti-platelet serum rendered thrombocytopenic animals. The level of those changes was lower than the level of corresponding changes reported by others in the irradiation thrombocytopenic model, and for equally severe thrombocytopenia. The possible reasons for this difference were evaluated and discussed.

C. The experimental technique developed here to study the vascular wall thrombocytopenic changes has certain advantages that renders it a useful tool in the study of platelet functions and in the evaluation of drugs claimed to have an anti-purpuric effect. One of the main attractions of this model is that it distinguishes between red blood cells extravasated through direct bleeding during the cannulation operations and spontaneously extravasated red blood cells post-operatively and post-APS. This is simply achieved by infusing the tagged RBC long after any possible bleeding has ceased and using those tagged RBC as a measure of RBC content in lymph. The animals are rendered thrombocytopenia post-the infusion of the tagged RBCs. Furthermore, the model has been shown to be reversible and platelets can be transfused and survive into the thrombocytopenic animals shortly after the anti-platelet serum infusion into the same animals.

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"AND IN THE BEGINNING IT WAS LIGHT,
ONLY NOBODY WAS THERE TO SENSE IT;
THEREFORE, IT DID NOT MAKE ANY
DIFFERENCE WHETHER IT WAS LIGHT OR
DARK.
AND THEN MAN CAME ALONG AND CREATED
CONFUSION."

- GREGORIOS

CHAPTER 1

EXTRAVASATION OF ERYTHROCYTES FROM THE INTACT VASCULAR COMPARTMENT INTO THE INTERSTITIAL SPACES IN POST-IRRADIATION THROMBOCYTOPENIA

1. Post-Irradiation Thrombocytopenia

The first published observation of the relationship between lethal range irradiation and thrombocytopenia was made by Lacassagne and Lavedan[1] in 1922.

Shouse et al[2] in 1931 related this thrombocytopenia to the post-irradiation (lethal range) hemorrhagic and purpuric tendencies in dogs. They also postulated that, since platelets are not sensitive to this range of irradiation, it was the severe damage of megakaryocytes and cessation of thrombopoiesis that caused the thrombocytopenia, as normal aging removed platelets from the circulation. From this hypothesis, and their measurements of post-irradiation platelet counts, they were able to calculate fairly accurately the life span of platelets in dogs.

2. Post-Irradiation Anemia. Escape of Erythrocytes from the intact Vascular Compartment

Over a quarter of a century ago it was a well known fact that in cases of severe total body irradiation of humans [3,4,5] and of animals[2,5,6,7] post-irradiation gen-

eralized purpura accompanied by hemorrhagic tendencies and severe anemia was one of the common homeostatic disturbances contributing to eventual death. This anemia is not always associated with macroscopic bleeding and it is of such steep onset and progress than it could not be explained at the time, even in the presence of complete cessation of hemopoiesis, in view of the fairly long life span of erythrocytes. Furthermore, this anemia was often accompanied by marked peripheral reticulocytosis indicating a still functional hemopoietic system.

Kahn et al[8] in 1952 were able to provide some experimental evidence that massive (700-1000 r) total body irradiation of animals (rabbits) causes a severe anemia without direct injury of erythrocytes*. They injected tagged RBC into some animals before irradiation and into other animals post-irradiation, and studied the disappearance rates, which were found to be the same, but faster than in control non-irradiated animals. They postulated that the observed anemia was caused in part by irradiation induced damage of the endothelium of the capillary wall, resulting in erythrocyte extravasation into tissue spaces and lymphatics. On the basis of their histological observations, they assumed that injury of the

* about 20,000 r are needed in vitro to produce a hemolytic effect

erythrocytes during the escape phase and during their movement through tissue spaces resulted in their subsequent removal by the reticuloendothelial system. In autopsies of sacrificed animals, depending on the stage of the post-irradiation syndrome, they found depletion of lymphoid elements and the presence of erythrocytes in lymph sinuses, and at later stages, hemosiderin in large mononuclear phagocytes in the lymph nodes and erythrocytes in the sinuses, and at even later stages, hemosiderin deposits in lymph nodes with only a few erythrocytes present.

Kahn et al concluded that the post-irradiation anemia is in part absolute, as there is impairment of erythropoiesis and in part relative due to the diversion of erythrocytes into the extravascular space and fluctuation in plasma volume. However, Kahn et al did not postulate any correlations between post-irradiation thrombocytopenia and its contributions to the altered physiological properties of the vascular wall.

Further evidence of increased capillary permeability in post-irradiation hemorrhagic syndrome was provided by Wish et al[9] in 1952, who studied the increased disappearance rate of varied tagged substances, including homologous and heterologous erythrocytes and plasma, from the blood stream of irradiated (LD-50) rabbits and mice. They found that 2-14 days post-irradiation (LD-50) these

substances disappeared faster from the blood stream of irradiated animals than from the bloodstream of control animals.

In a concurrent sudy to that of Wish et al, Bigelow et al[10] sampled thoracic duct lymph of irradiated dogs and cats and studied its cellular and chemical composition changes. They found an increased flow of lymph and an increased red blood cell content in the lymph of the irradiated animals. This increase seemed gradual past the second post-irradiation day, reaching a maximum of about seven to fourteen days. They reported, for eight animals, an average maximum RBC count in lymph of 523.2 x $10^3/mm^3$ (23.5-1,070x$10^3/mm^3$), up from a normal average value of 0.31x$10^3/mm^3$. They also concluded that post-irradiation anemia is due to the diversion of erythrocytes into the lymphatics and tissue compartment and that many of the extravasated erythrocytes are destroyed. Bigelow et al did not make any correlations between thrombocytopenia and increased capillary permeability at this stage.

But, in a later publication of the same group (Ross et al[11], 1952) they make the important observation that, since massive localized irradiation is not followed by hemorrhage at irradiation sites, the possibility of direct endothelial cell injury as the cause of the observed post-whole body irradiation increased capillary

fragility and permeability is rather unlikely. Instead, Ross et al preferred to choose in favour of Cronkite's et al[5], 1952, theory of thrombocytopenia related capillary fragility and increased permeability. They also considered possible, as a less favoured hypothesis, that direct bleeding into degenerating tissue of lymph nodes may be a contributing factor for the appearance of increased red cells in the lymph of the thoracic duct of the irradiated animals.

3. The General Coagulation Defect of the Post-Irradiation Syndrome and the Role of the Platelets

Rosenthal et al[13] in 1950, in their study of the blood coagulation impairment in the post-irradiation hemorrhagic syndrome, postulated that the post-irradiation coagulation defect can be attributed chiefly to diminished platelet levels.

Cohn[14], in 1952, also relates the coagulation defect to the post-irradiation decreased levels of circulating platelets and underlines the similarity of the clinical anatomical picture of the post-irradiation syndrome to that of thrombocytopenic purpura. His experiments demonstrated that the blood coagulation defect in irradiated animals and in particular the rate of clot retraction, correlated well with platelet level

and that clot retraction ceased to occur below a blood platelet count of about $38 \times 10^3/mm^3$.

But it was Cronkite, Brecher and their associates [5,14-17], 1949-1952, who eventually provided direct evidence that the main cause of the post-irradiation hemorrhagic syndrome was thrombocytopenia and that there was no evidence of other impairment of the then known hemostatic mechanism. They were able to demonstrate that transfusions of fresh platelets could rectify the failure of the irradiated animals to utilize prothrombin and the subsequent impairment of blood coagulation mechanism in vivo and in vitro. Also, by daily transfusion of fresh platelets, before thrombocytopenia developed in irradiated animals ($\geq$ LD50) they were able to prevent the hemorrhagic phase as seen at autopsies of the sacrificed or succumbed animals.

4. <u>The Role of Platelets in Maintaining a Competent Intact Vascular Compartment</u>

Woods et al[18] in 1953, in an extension of their previous work[10], described the extent to which platelet transfusions diminish the hemorrhagic tendency in irradiated dogs and rats as manifested by increased erythrocyte counts in lymph. They postulated (see also 11, 15, 19) that platelets exercise a role in preventing

or repairing endothelial damage, perhaps by contributing to the actual formation of inter-endothelial "cement substance" and that lack of the latter is the real cause of the increased spontaneous escape of red cells from the vascular compartment in thrombocytopenia. No other sub-cellular fraction they tried (such as fresh rat-brain thromboplastin) could duplicate the effect of intact platelets in rectifying the vascular wall impairment in the irradiated thrombocytopenic animals and thus reducing red cell concentration in lymph.

Jackson et al[20] (1959) reiterated the findings of Woods et al on the effectiveness of transfusions of fresh platelets in increasing platelet counts in blood and in suppressing red cell counts in the lymph of the post-irradiation thrombocytopenic animals.

Furthermore, they found that lyophilized platelets transfusions are ineffective in increasing platelet counts in blood and in reducing red cell counts in lymph.

It was Roy et al[21], in 1972, who first studied simultaneously the effect of fresh platelet transfusions, in post-irradiation thrombocytopenic animals, on both the "spontaneous escape" of formed blood elements from the intact vascular compartment and on the impairment of the platelet hemostatic plug formation and coagulation defect.

They found that larger platelet concentrations in blood are needed to rectify coagulation defects, whereas even small transfusions of fresh platelets that did not have a significant effect on platelet counts would bring a striking reduction in throacic duct lymph red cell counts. They reiterated the postulation that separate mechanisms of action account for the effects of platelets on maintenance of endothelium and vascular wall competency and on bleeding from mechanically injured vessels.

5. Red Cell Counts in Lymph of Irradiated Thrombocytopenic Animals. An Inventory

Other than the previously mentioned research workers, Aursness[22,23] (1973) also studied the changes in cellular content of the lymph and changes in vascular permeability as a function of post-irradiation thrombocytopenia. In his experiments Aursness chose to sample the peripheral lymph from the ear lymph vessels and before it passes through any lymph nodes. Thus, the increased red cell counts in lymph that he observed were the result of generalized extravasation of red cells and not the result of direct entry at lymph nodes and lymph-venous anastomoses. Also, the lymph he collected did not originate in special tissues with fenestrated vascular endothelium, such as liver and spleen (see Ref. 24) and red blood cells in

this lymph could not have gained entry into the lymph following extra-vasation in sites of mechanical capillary injury. In seven out of eleven animals he observed an increase in lymph red cell count which coincided with the most marked thrombocytopenia level of below 10,000 plat./ mm^3. This increase was strikingly less than the one reported by the previous mentioned workers who were sampling the thoracic duct lymph, and also occurred at lower blood platelet levels. In another four animals with comparable severity of thrombocytopenia, no such increase of lymph R B C levels was observed. Aursness attributed this difference to the possibility that young, hemostatically more efficient platelets than the old ones were already circulating in the latter four animals and although they were not in large enough numbers to cause a measurable increase in blood platelet levels, they were sufficient to repair the endothelium and establish normal vascular wall competency. This is in agreement with Roy's et al observation (see above). Aursness also observed that in the seven animals that presented with increased R B C levels in lymph, the increase was usually rectified at about one day before an increase of blood platelet levels was measurable, as perhaps the first new platelets coming into circulation were consumed in

repairing endothelium damages before an increase in numbers could be sensed.

CHAPTER 2

IMMUNOLOGICALLY INDUCED THROMBOCYTOPENIA

1. Other Models for the Experimental Induction of Thrombocytopenia

Other than whole body irradiation, there are three main techniques for the experimental induction of sustained thrombocytopenia or the reduction of blood platelets in thrombocythemic patients.

i. Thrombocytopheresis[25,26] which is the mechanical removal of platelets from blood via repeated exchange transfusions with platelet-poor blood either from a donor[26] or from the patient.[25] A quantity of blood is drawn from the patient and it is differentially centrifuged for removal of platelets. The platelet-poor blood is transfused back into the patient. This sequence is repeated several times until a satisfactory blood platelet level is achieved.

ii. Impairing platelet production with cytotoxic drugs such as Busulphan, a sulphonic acid ester that acts as alkylating agent. These drugs can cause bone marrow aplasia, and subsequently thrombocytopenia in animals and in patients.

iii. Immunologically, by injecting into the

animals an heteroimmune anti-platelet serum (Mattison[28]). The latter of the above models is pertinent to this thesis and we next review it to some extent.

2. A Review of Immunologically Induced Thrombocytopenia, by use of an Heterologous Anti-platelet Serum

The idea and the actual production of an heterologous anti-platelet serum by immunization of an animal with heterologous platelets, and the subsequent use of blood serum from the immunized animal to induce thrombocytopenia when injected into the platelet donor species, dates back some seventy years. Marino[29] in 1905, LeSourd and Pagniez[30,31] in 1906-1908 and Cole[32] in 1907 are reported to be the first investigators to develop this technique.

It was shown by some of the early investigators in this field[31-36] that these heterologous anti-platelet sera were definitely species-specific and in vitro capable of aggultinating platelets, and in the presence of complement lysing them to a certain extent. Similarly, these anti-platelet sera were found to agglutinate and lyse red cells homologous to the platelets, for which they are specific. Conflicting reports[34-36] about successful and unsuccessful efforts to demonstrate cell specificity of

these early sera, by means of absorption experiments, made the question of cell specificity doubtful at that time (see also below, Bedson[43,44]). Ledingham[37] in 1914 was able to demonstrate that anti-guinea pig platelet serum, other than diminishing circulating platelet levels, had a toxic effect on the guinea pig, inducing in this animal a condition closely resembling purpura haemorrhagica in man. A year later, Ledinghma and Bedson[38] demonstrated that hetero-immune anti-red blood cell and anti-polymorphonuclear leukocyte sera, although also toxic to the animals (as the anti-platelet sera), did not produce the pathological picture of purpura haemorrahagica induced by the anti-platelet sera. Lee and Robertson[39] in 1916 and a number of other investigators[40-42] reinforced these observations of Leningham and Bedson and gave weight to the hypothesis that depleted levels of circulating platelets and toxic damage of the vascular compartment were needed for the appearance of purpura.

Bedson[43,44] in 1922 shed some light on the question of cell specificity of blood elements anti-sera. He produced anti-platelet sera free from red cell agglutinins (in vitro) by incubating anti-platelet sera with red cell suspensions and he used this assumed platelet specific anti-serum to induce thrombocytopenia and

purpura in guinea pigs. He concluded that damage and agglutination of red cells was not the reason of the appearance of purpura in anti-platelet serum produced thrombocytopenia.

Of interest is the hypothesis formulated by Bedson in his discussion (although not solidly supported by a strong experimental basis at that time); we quote: "It would seem that in purpura, produced by means of an anti-platelet serum, the sequence of events in the production of haemorrhages is as follows: the capillary endothelium is first of all damaged, and later, when the platelets have been greatly reduced in number in the circulating blood, a leakage of red cells (by what appears to be an exaggerated form of diapedesis) through the capillary wall into the surrounding tissues takes place. Had the platelets been present, hemorrhages would not have occurred, because their presence in the outer more slowly moving stream, together with the leukocytes, would have prevented the rapidly moving central stream of red cells from coming in contact with the endothelium and of finding out its weak spots. They (platelets) would also have helped in making good the deficiencies in the vessel wall caused by the endothelial damage."

Since then, a number of works have been published on the cytological and physiological sequences involved in anti-platelet serum induced thrombocytopenia and its sequellae of purpura[26,28,45,54] and the model has been used to study a variety of problems related to megakaryocytopoesis, for the production of thrombopoietin-rich sera, the study of thrombocytopenic conditions and also in the evaluation of the role of platelets in the process of a variety of diseases.[55,56]

In the above quick review, we deliberately chose not to dwell on specific aspects of homogogous and autologous anti-platelet antibodies and on human thrombocytopenic/thrombocytopathic diseases with an immunological basis, as this would have been well beyond the scope of this thesis. A good review of immunologic mechanisms of platelet damage (up until 1972) is given by Osler et al.[51] We also recommend Williams et al[57] hematology textbook and the review article by Hirsh et al.[58]

3. <u>How APS Induced Thrombocytopenia relates to our Research Proposal</u>

The previously discussed model has been used on two occasions[53,54] to study, via electron microscopy, the qualitative increase in permeability of the vascular endothelium and vascular wall to red cells, in the presence

of severe thrombocytopenia (see also Chapters 3,4). However, no quantitative studies of the vascular permeability increase in anti-platelet serum induced thrombocytopenia has been done as yet. In this thesis, we propose to do this by direct sampling of thoracic duct lymph of rabbits and evaluate (measure) changes in the red cell concentration and red cell flux in their thoracic duct lymph as a sequel of APS induced thrombocytopenia. A comparison of our results with those discussed in Chapter 1 (irradiation model) could be useful in understanding better what brings about the increased rate of spontaneous extravasation of red cells in thrombocytopenia, and to what extent this is due to the actual lack of platelets, or if this lack of platelets is simply unmasking actual damage inflicted on the endothelium by the means used to induce thrombocytopenia (see results and discussion chapters).

CHAPTER 3

THE ENDOTHELIUM AND ITS INTERACTIONS WITH PLATELETS AND RED CELLS

This chapter is not meant to be a comprehensive description of structure and function of capillaries and of endothelial cells; we simply give here certain definitions and descriptions that are pertinent to understanding our results and discussion chapters.

1. Classification of Blood Capillaries and their Endothelium in Mammals

The classification of blood capillaries and endothelium used here is the one described by Majno[24] (1965). Although in reality there are almost as many types of capillaries as there are organs, he distinguishes three main types of capillaries and his classification is based on the cellular structure and continuity of their endothelial layer as it appears on electron microscopic studies.

We next present Majno's description.

i. Type I or continuous capillaries have continuous sheet of tightly connected endothelial cells and a continuous basement membrane. This type of capillaries is found in striated muscle, myocardium, smooth muscle of digestive and reproductive system, lung, central nervous

system, subcutaneous and adipose tissue, placenta, dermis. Their endothelium is about 0.15 to 0.3 μ thin but it bulges 2 to 3 μ at the site of nucleus. The cells include small number of mitochondria, ribosomes, lysosomes, scanty endoplasmic reticulum and many vesicles[24,54]. These vesicles, although found in other cells also, are by far more numerous in endothelial cells and sometimes their total volume makes about one third of the cell volume[59]. They are thought to be part of the transport system of the cells and under normal conditions, do not bridge the entire thickness of the cell.

In type I capillaries, endothelial cells are flat and tend to assume an hexagonal shape. Occasionally, their lumenal surface shapes into small projections and folds.

This type of endothelium is particularly suited to allow passage of nutrients, yet remains impermeable to blood cellular elements or the bulk of plasma proteins.

ii. Type II capillaries or fenestrated capillaries are similar to type I in completeness of endothelium sheet and basement membrane, but differ in endothelial cell shape and structure.

Their endothelial cells are thinner (often as thin

as 200 to 400 Å) and seem to be pierced with fenestrae, which can be either open or closed with cellular membrane. These fenestrae are about 0.1μ or less in diameter. Another difference is that these endothelial cells contain by far fewer vesicles than those in type I capillaries. Capillaries of this type are found in the renal glomerulus and tubules, ciliary body of the eye, choroid plexus, intestinal mucosa villus, exocrine and endocrine glands, gall bladder, renal medulla and synovial membrane. These are tissues specialized for the rapid exchange of fluid and solutes, and it seems that fenestrated endothelium function is to allow increased vascular permeability to fluid and solutes, but not to blood cellular elements, (for morphometric data on type I and type II endothelium see Simionescu et al[60] (1974)).

iii. Type III or discontinuous capillaries, have intercellular gaps between endothelial cells and discontinuous or absent basement membrane. Under Majno's classification, we refer to these capillaries as (true) sinusoids. They are found in organs which have a primary function of pouring into the blood or removing from it whole cells, large molecules and foreign particles. Such organs are bone marrow, spleen and liver.

2. Irradiation Injury of Endothelial Cells

Although some of the early researchers considered the possibility that direct injury of endothelial cells from irradiation was partially responsible for the post-irradiation hemorrhagic syndrome (Ross et al[11], 1952) they eventually argued against it on the basis that endothelial cells divide slowly under normal conditions. Therefore, these cells should be less prone to severe direct irradiation injury than the fast dividing red bone marrow cells, and any injury should have a very long latent period. Their postulation was further supported by the fact that localized massive irradiation is not followed by hemorrhages at irradiation sites. However, they acknowledged the fact that not much was known at the time about the life span and turnover of endothelial cells.

Since then, some indirect evidence has been provided that endothelial cells do actually suffer irradiation damage[61-63]. Direct evidence was provided by De Gowin et al[64] (1974), who were able to demonstrate that cultures of human endothelial cells, growing in vitro by the method developed by Lewis et al[65] (1973), were sensitive to irradiation.

They measured both deoxyribonucleic acid synthesis by the uptake of tritiated thymidine and replication of endothelial cells by direct counts, and found that

the growth of replicating endothelial cells seeded in flasks was completely inhibited for at least a week following an 870 rads irradiation dose. Smaller irradiation doses elicited a dose-response relationship. Residual effects persisted for at least two weeks. On monolayer cultures, irradiation doses of 1,750 rads caused minimal attrition, but still there was inhibition of replication.

Also, an 870 rads irradiation dose, on flasks containing nonreplicating confluent endothelial cell cultures, seemed to damage the mitotic apparatus of the cells, even though the cells were not dividing at the time of irradiation. When some of these cells were transferred to other flasks, they failed to increase in numbers or label with ^{3}HTdR, in contrast to the strong multiplication of the unirradiated controls. However, these cells were still living as manifested by viability tests.[64]

The irradiation dose range and the results of De Gowin et al are congruent with the work of Reinhold[62] (1972) who studied the irradiation dose-response of the in vivo growing rat capillaries.

3. Shared Antigenicity of Platelets and Endothelial Cells. Their Response to each other's Anti-Sera

Bedson[44] in 1922 (see also above) observed that transient lowering of the platelet level in guinea pigs by use of agar serum did not produce purpura as it did when he induced thrombocytopenia by use of anti-platelet serum. He also observed that anti-red cell serum alone did not produce purpura, although it may have had an immunologically injurious effect on platelets and endothelium. However, the combination of agar serum to induce thrombocytopenia and anti-red cell serum to cause endothelial cell injury resulted in hemorrhagic purpura signs (as observed in autopsies). He deduced that the antiplatelet serum (and the anti-red cell serum) were damaging endothelial cells as well as platelets (see his full hypothesis above).

Katsura[66] (1923) has been reported (original paper in Japanese) by Clark et al[67] (1950) to have demonstrated that anti-dog platelet serum agglutinated endothelial cells in vitro and fixed complement when reacted with endothelial cells. He also demonstrated that anti-endothelium cell serum, prepared by use of aortic endothelium scrapings as an antigen, could in vitro agglutinate platelets and in the in vivo tests, produce purpura

in the absence of measurable thrombocytopenia.

Contrary to the Bedson hypothesis that thrombocytopenia plus endothelial cell injury are needed for the appearance of purpura, Katsura demonstrated that anti-platelet serum absorbed with endothelial cells in vitro could still induce thrombocytopenic purpura in vivo. This is in agreement with the research of Gauldie et al (see below) and with our work. However, we question whether it is possible to remove all the anti-endothelial cell activity from APS serum.

Katsura also induced severe hemorrhagic purpura in animals by combining a subpurpurigenic dose of anti-platelet serum with a subpurpurigenic dose of anti-endothelium cell serum, thus indicating the synergism of these sera.

Clark et al[67], using rabbit anti-dog endothelium serum, induced severe purpura in dogs without concurrent thrombocytopenia, but contrary to Katsura's work, he found that the in vitro tests of his anti-endothelial cell serum for platelet antibodies were negative; this difference was perhaps due to the different serum titer for anti-platelet antibodies in the sera used by these two investigators.

Indirect indication about the common antigenicity

of platelets and endothelium is also provided by the works of Cronkite et al [68,69] (1957, 1961), Johnson et al[70] (1964) and Worcik et al[71] (1969). These investigators were able to provide some direct evidence (see below) that platelets interact with endothelium and donate some membrane material and cytoplasm to endothelial cells as part of their role in what Rebuck[72] (1963) described as their endothelium supporting function.

It is rather reasonable to assume that the transfer of platelet membrane and cytoplasm material also transfers their antigenic substances and that consequently, endothelial cells may indeed have many common antigens with platelets.

Hagel et al[73] (1962) sensitized dogs with homologous platelets prior to renal transplantation. An accelerated rejection of renal transplants, as compared to controls, was observed in these animals. The prominent histologic finding in the damaged kidneys was vascular damage.

Morrison et al[74] (1969) repeatedly injected (subcutaneously) dogs with homologous aortic endothelium antigen and studied the survival of homologous platelets transfused into these animals. No change was observed in the survival of autologous platelets, but a significant

decrease in the survival of homologous platelets was demonstrated. However, serologic tests in vitro could not detect platelet antibody in the serum of the immunized animals.

Becker et al[75, 76] (1969, 1973) were able to demonstrate, by direct and indirect immunofluorescence techniques, that rabbit antisera to human platelet contractile protein thrombosthenin stained mature megakaryocytes, blood platelets, endothelial cells of arteries, arterioles, veins, venules, liver sinusoids and capillaries of the heart and skeletal muscle. The endothelial cells of veins appeared to stain more intensely than endothelial cells of capillaries, indicating that perhaps venous endothelium is more contractile than that of capillaries.

Recently, Gauldie[77] was able to remove antiendothelial cell activity from antiplatelet antisera, as manifested by immunofluorescence techniques, by absorption with endothelial cell preparations. These anti-sera still induced purpura in animals, as manifested by our direct sampling of thoracic duct lymph for red cell content (see results and discussion chapters).

4. Platelet Support of Endothelium

The role of platelets in maintaining an intact

functional vascular compartment that prevents unwarranted loss of cellular and molecular blood elements is twofold:

a) participation in the arrest of bleeding from injured blood vessels;

b) Maintenance of an intact functional endothelium (and perhaps supporting structure) that possesses certain desirable permeability characteristics to cellular and molecular elements in blood.

The mechanism and biochemistry of the former of the above platelet functions and its deviations from normal have been well investigated; any standard hematology textbook gives an ample account of this function of platelets. An excellent account is given in a review by Mustard et al[78] (1971).

The latter of the above platelet functions, which is pertinent to this thesis, is not yet fully understood. We next discuss what is known and the current predominant theories to explain this platelet function.

There are basically two postulated mechanisms on how platelets maintain vascular endothelium integrity:

i) that platelets interact with and donate membrane material to endothelial cells;

ii) that platelets adhere to the exposed basement membrane where the endothelium has been injured or where endothelial cell contraction or vasodilation has simply caused gaps to appear between endothelial cells.

The first of the above theories is supported by the works of Cronkite et al[68,69] (1957), Johnson et al[70] (1964) and Wojcik et al[71] (1969). Their studies were made of thrombopenic subjects transfused with fresh platelets. They postulated that under these experimental conditions the phenomenon would be amplified, because the uptake of platelets material from "starved" endothelium would be manifold times that of normal conditions.

Cronkite et al transfused homologous $S^{35}O_4$ labelled platelets into irradiated thrombopenic rats and into normal controls and did historadioautographs of the capillary beds of the animal tissues. In thrombocytopenic animals, a consistent picture of apparent labelling of capillary beds was found. They postulated that perhaps either intact platelets or radiosulfur-labelled material (possibly mucopolysaccharides) from the platelets adhered to and lined the thrombopenic endothelium.

Johnson et al did electron microscopic studies of capillaries in the dermis of severely thrombocytopenic

patients following fresh platelet transfusions, and they interpreted some of their electron micrographs as depicting platelets in the process of incorporation into the endothelial cytoplasm.

Wojick et al transfused irradiation thrombocytopenic guinea pigs with tritiated diisopropylfluorophosphonate labelled homologous platelets. They did electron autoradiographic preparations of tissue taken at various intervals following the transfusion and they found label being localized in endothelial cells soon after the transfusion. Furthermore, many platelets were observed interacting with endothelium immediately after the transfusions.

On the basis of their autoradiographic findings, they described a possible process by which whole platelets are incorporated (not phagocytosed) into endothelial cells donating both membrane material and cytoplasm.

Another indication for the endothelial nourishing role of platelets is provided by the work of Saba et al[79] (1975) who demonstrated that platelets or platelet components, such as ADP and serotonin, significantly enhanced the growth of cultured human endothelial cells, the whole platelets having the maximum effect (+87%) under the conditions of these experiments.

The second of the two postulated mechanisms on how platelets support endothelium, namely that platelets adhere to the exposed basement membrane, where endothelial cell contractions or minimal damage has caused gaps to appear between endothelial cells, has been postulated by Brecher and Cronkite[15] (1951) and Woods et al[18] (1953). They suggested that platelets contribute materially to the formation of inter-endothelial "cement" substance , and that in thrombocytopenia it is the lack of that material that results in loose junctions between the cells, thus allowing for the extravasation of red cells and plasma proteins.

However, most of the direct evidence for this theory has been provided by Tranzer et al[80] (1967), who did electron microscopic studies of capillaries of animals pretreated with reserpine, and of animals with sympathetic denervation. In contrast to control animals, where the endothelium was found to completely cover the underlying basement membrane, gaps appeared between the endothelial cells of the sympathetomized animals and of those which had been treated with reserpine. Furthermore, platelets (but never blood cells) seemed to have made contact with this exposed basement lamina via pseudopods. Larger exposed areas were covered by larger numbers of

platelets, but no signs of platelet thrombic aggregation such as degranulation or fibrin formation were present.

Tanzer et al suggested that this interaction between basement lamina and platelets was possibly mediated through electrical forces.

Some indirect support for this model of platelet support of endothelium is rendered from the work of Ohsaka et al[81] (1975). They did electron microscopic studies of the vascular endothelium of rats treated with the venom of a crotalid that has been shown in vitro to impair platelet function (aggregation). They observed opened up intercellular junctions (some up to 0.7μ wide) with red cells spurting through them, while platelets could be seen in circulation. It could be postulated that the impaired platelet function resulted in loss of their ability to provide for "cement" substance for the endothelium bed, and consequently resulted in loss of tightness for the inter-cellular junctions. However, other interpretations may be equally plausible.

Gore et al[53] (1970), in electron microscopic studies of the microcirculation of anti-platelet serum thrombocytopenic guinea pigs, were able to observe focal junctional separation of endothelial cells in capillaries and venules; these were accompanied by gaps and disruptions

of adjacent portions of the basement membrane. Both RBC and tracer particles were seen within those junctional separations.

However, the above observations were not supported by the findings of Kitchens et al[54], Johnson et al[70], Wojcik et al[71] and Van Horn et al[82,83] (see below). None of these investigators observed intercellular junctions which had opened up, in their electron microscopic studies of capillaries of thrombocytopenic animals.

Whether the platelets are supplying "cement" substances to maintain tight intercellular endothelial cell junctions, or are maintaining endothelium by being wholly or partially incorporated into endothelial cells, the in vivo evidence up until now indicates that whole functional platelets are needed to perform this function. Efforts to reverse or suppress the purpuric picture of severe thrombocytopenia via infusion into thrombocytopenic animals of freezed and lyophilized platelets, or platelet sub-cellular fractions or other biologically active molecules, have failed.[15,18,20,84] Also, in vitro[79], although platelet substances such as ADP and serotonin have been shown to have an accelerating effect on growing endothelial cell cultures[79], the effect of whole platelets is significantly superior, indicating that they probably affect

endothelial cells in a multitude of ways.

The effectiveness of platelet rich media in maintaining vascular integrity has also been demonstrated in a number of organ perfusion studies in vitro[85-87]. Gimbrone et al[87] perfused each of the two lobes of dog thyroids with autologous platelet rich plasma and the other with autologous platelet poor plasma. The lobes perfused with the platelet poor medium showed increased extravasation of RBC and albumin, as indicated by the increased trapping of Cr^{51} labelled RBC and I^{125} labelled albumin, when pulses of blood were sent through the organs at the end of the perfusion period.

The platelet poor plasma perfused lobes showed on macroscopical examination oedema, capsular hemorrhages and purpuric follicles, and on electron microscopic studies, changes that varied from focal cytoplasmic swelling, to necrosis of endothelial cells.

CHAPTER 4

VASCULAR WALL PERMEABILITY CHANGES AND THE EXTRAVASATION OF ERYTHROCYTES FROM MACROSCOPICALLY UNINTERRUPTED CAPILLARIES AND VENULES

1. Introduction

Increased vascular permeability to cellular and subcellular blood components (8, 9, 10, 11, 18, 21, 22, 23, 38, 39, 44, 53, 54, 70, 71, 82, 83,) is one of the two cardinal manifestations of severe thrombocytopenia, the other being the impairment of normal hemostatic mechanism. However, thrombocytopenia per se, without concurrent insult to the vascular compartment wall, is not fully proven to cause an increased rate of the spontaneous extravasation of erythrocytes. Furthermore, spontaneous extravasation of erythrocytes and/or of subcellular plasma components can be brought about in the absence of thrombocytopenia and by a number of methods. Some of these methods are: i. anti-endothelial cell serum (Katsura[66]); ii. certain snake venoms (Ohsaka et al[81], McKay et al[88]); iii. vasoactive substances such as histamine type mediators (Majno et al[89,90]).

In the three examples of non-thrombocytopenic vascular leakage mentioned above, it is not fully

understood yet if and how the introduction of those new variables (anti-endothelium cell serum, snake venom, histamine) affects the ability of platelets to interact with endothelium, so that even in the presence of normal platelet levels we may have a deficit in certain facets of their function.[81] Similarly, in experimentally induced thrombocytopenia, be it irradiation induced or anti-platelet serum induced, the means of inducing thrombocytopenia also affects the vascular endothelium and its supporting structures, both directly and indirectly.

Both irradiation and antiplatelet sera are possibly insulting endothelium directly. Furthermore, the heterologous anti-platelet serum influences vascular permeability indirectly as well, through the release of the vasoactive amines histamine and serotonin, from platelets affected by the serum. This release reaction has been demonstrated both in vivo and in vitro by Henson[92,93] (1970) and has a transient effect in increasing vascular permeability and lymph flow rates.[89, 91]

2. Platelet levels in experimental Thrombocytopenia. A comparison between the Irradiation and the Antiplatelet Serum Models

Although antiplatelet sera affect the megakaryocytes and thrombopoiesis (Rolovic et al[26], 1970), amounts of antiplatelet sera used to induce almost complete

thrombocytopenia, do not arrest thrombopoietic activity. Thus, in anti-platelet serum induced complete thrombocytopenia, new platelets come into circulation shortly after treatment[28,46,94] and the average age of these platelets is young. Contrary to this, in irradiation induced thrombocytopenia, there is cessation of thrombopoietic activity, and thrombocytopenia is achieved as the number of platelets dwindles away without new platelets coming into circulation. Thus, by the time thrombocytopenia is achieved, the average platelet age is old, unless there is already some recovery of bone marrow and resumption of thrombopoiesis. Therefore, if young platelets are indeed hemostatically more efficient as Karpatkin[95] (1972) has shown and if the same applies for their efficiency in maintaining endothelium as Aursnes[22] (1973) has postulated, then comparable levels of circulating platelets in the two thrombocytopenic models ought to result in smaller vascular permeability increases for the anti-platelet serum model. The same consideration would apply between irradiation thrombopenic animals in which some thrombopoietic activity had resumed, or it had not ceased completely, and those with more complete bone marrow damage. Thus, in experimental thrombocytopenia, comparable circulating platelets levels may

not be biologically comparable.

3. The Basement Membrane Defect in Experimental Thrombocytopenia

Currently, there is inadequate understanding of the role of the basement membrane defect[53] if any[54] in the increased vascular permeability in thrombocytopenia. The basement membrane changes in thrombocytopenia could be part of a more generalized physiological response, or merely a reflection of the impaired function of endothelial cells, since endothelial cells possibly do contribute in the production and organizing of basement membrane material[24,96-98].

4. Thrombocytopenic Permeability Changes of Capillaries and Venules

We previously discussed the fact that endothelial cells of capillaries vary from organ to organ, both in normal structure and in normal function, and that three main types of capillaries can be distinguished at the electron microscopic level (Majno[24]). Since the normal permeabilities of Type I, Type II, and Type III capillaries endothelium are different, we assume that their permeability changes in severe thrombocytopenia could also be different. Therefore, it is necessary for any meaningful comparisons of cellular and/or molecular thrombocytopenic permeability changes of the vascular wall, to refer

to the same type of capillaries. If the permeability changes are studied via sampling of lymphatics for cellular and molecular constituent changes, lymph vessels draining areas with comparable capillary structure should be utilized.

At the ultramicroscopic level, one must distinguish between cellular and molecular permeability of true capillaries as defined by Majno[24] (diameter not exceeding that of RBC) and the permeability of post-capillary vessels of capillary thinness but larger diameter which behave like venules. Venules are physiologically more permeable than capillaries and, as Majno[24,90,99,100] (1961-1969) has demonstrated, more susceptible to vaso-active substances and permeability changes. They are also more susceptible to immunologic injury (Movat et al[101] 1963).

Becker et al[76] (1973) demonstrated that endothelial cells of venules possibly contain larger amounts of contractile proteins, such as thrombosthenin, than endothelial cells of capillaries. This is suggesting that venous endothelial cells are more contractile than those of capillaries. According to Becker, this may be partly responsible for the different permeability changes of venules and capillaries endothelium in response to

treatment with vasoactive amines[24], since the stronger contraction of endothelial cells of venules would lead to the opening up of their intercellular junctions earlier and to a larger extent than intercellular junctions between endothelial cells of capillaries.

The thinning of endothelium in thrombocytopenia[76] in conjunction with the fact that the tangential forces in the wall of the venules are bigger than in the wall of capillaries* could also result in the intercellular junctions in venules reaching their threshold tangent strength and open up before the endothelium cell junctions in capillaries do so.

However, the question of differential opening-up of inter-endothelial cell junctions of venules and of capillaries in thrombocytopenia has not been answered yet. Furthermore, if indeed there is a widening or opening-up of intercellular junctions, we are not sure yet as to what extent this is contributing to the increased vascular permeability in thrombocytopenia (see below).

* Laplace Law: $T=Pr$, where T is the tension of tangential stretching force per unit length of vessel in dynes/cm, P is the lateral distending pressure in dynes/cm^2 and equals the intraluminal pressure in excess of the surrounding tissue pressure, and r is the vessel radius in cm.

5. The Mechanism of the Extravasation of Cellular and Sub-cellular blood components from Macroscopically intact Capillaries and Venules. Qualitative Electron Microscopic Studies

Van Horn et al[82,83] (1966, 1968) studied, via electron microscopy, the spontaneous extravasation of erythrocytes in irradiation thrombocytopenia and the extravasation of colloidal carbon particles both in irradiation thrombocytopenia and in anti-platelet serum induced thrombocytopenia. They reported an increase in the permeability of the capillaries and venules wall to red blood cells and to carbon particles. The latter seemed to escape from the microvessels through what would appear to be a process of entrapment into invaginations in the endothelial cytoplasm and transport via vesicles through the endothelium. The endothelial cell junctions were intact both in capillaries and in venules and carbon particles were not seen inside the junctions. They also reported that the observed extravasation of RBC in thrombocytopenia was apparently a transcellular one, the RBC seemingly penetrating through the weakened endothelium cytoplasm and not through intercellular junctions. Although Van Horn et al did not make a clear distinction between capillaries and venules in their discussion on the extravasation of erythrocytes, one can judge from their

published electron-micrographs that both capillaries and small venules are similarly involved (see also Ref. 70, 71).

Similar to this were the observations of Kitchens et al[54] (1975) who studied via E.M. the ultrastructural changes of Type I endothelium in anti-platelet serum and in Busulphan treatment rendered thrombocytopenic rabbits. They also did not observe any major junctional separations. However, they did observe thinning of the endothelium, the appearance of transcellular fenestrae, a reduction in the number of vesicles in the cellular cytoplasm, and a disappearance of the normal projections and folds of the lumenal surface of this type of endothelium. There was an increase in vascular permeability manifested by a prominent extravasation of RBCs and tracer colloidal particles (thorotrast), apparently through a transcellular mechanism. No tracer particles were observed in intercellular junctions or RBC transversing the endothelium layer via opened-up junctions. Their postulations for a transcellular extravasation mechanism for blood components through the vascular endothelium in thrombocytopenia was the same as Van Horn's et al.

In contrast to the above, Gore et al[53] (1970) in

his ultramicroscopic studies of myocardium capillaries in anti-platelet serum rendered thrombopenic guinea pigs, reported intercellular junction openings in the lining of microvessels exceeding 8μ in luminal diameter. The basement membrane beneath the opened-up junctions was also disrupted. Erythrocytes and tracer particles seemed to escape through the opened-up junctions.

However, whether the extravasation of cellular and subcellular blood components in thrombocytopenia was studied qualitatively via electron microscopy or quantitatively via sampling of the lymph for cellular and subcellular content changes, the defect was always rectified shortly (within minutes to 2 hours) post-infusion of viable fresh platelets or following a spontaneous increase in circulating platelet levels[18,20-23,53,54,70,71,84].

CHAPTER 5

MATERIAL AND METHODS

1. Animals and Operational Procedures

a. Animals: i. Rabbits. New Zealand white rabbits raised with standard Purina Rabbit Chow (0.004% sulfa quinoxaline content to prevent quoxidiosis) and water ad libitum; animal weight, $\sim 2.48 \pm 0.28$ Kgr;

ii. Guinea pigs. Random bred guinea pigs raised with standard Purina guinea pig chow and water ad libitum; animal weight, 400-800 grs.

b. Anaesthesia: rabbits were kept anaesthetized throughout the duration of the experiments with intra-arterial infusion of 1 part ~~sodium~~ pentabarbitol (65 mg in 1 ml) solution in 9 parts physiologic (0.85%) saline. Sodium pentabarbitol content of final dilution was 6.5 mg in 1 ml solution. Usually, 1.0 ± 0.3 ml of this solution, per hour, per kilogram of animal weight was required to maintain sufficient levels of anaesthesia. Sodium pentabarbitol preparation used was Somnotol (100 ml vials) manufactured by MTC Pharmaceuticals, Hamilton, Ontario. This preparation consists of sodium pentabarbitol 65 mg/ml and 1% benzyl alcohol as a preservative in an aqueous

propylene glucol base.

c. Cannulation of the thoracic duct: This was done just cephalad to the point where the duct originates from the Cisterna Chyli, according to the method of Bollman et al[108] (1948) as modified by Bienenstock and Associates.[109] The main modifications employed here are as follows: i. the tip of the polyethylene cannula inserted into the duct is preformed into an L shape. This is to avoid the unwarranted bending of the previously used straight cannulas that could result either on a cut off of lymph flow or in the tip of the cannula penetrating the very thin wall of the duct. Shaping of the polyethylene tubing was easily accomplished over hot steam; ii. The point of insertion of the cannula and surrounding area was carefully dried with gauze and a thin layer of Eastman 910 tissue adhesive (manufactured by Armstrong Ltd., Montreal, Quebec) was applied to completely seal the point of insertion. When this first layer dried, a second application of this fast bonding adhesive was applied on the wall of the aorta, over the cisterna chyli wall and on the muscles posterior to the duct at the point of cannulation (quadratus lumborum). The animal tissues displaced during the operation were allowed to come back in place and when the adhesive dried (15 min) the cannula and duct were protectively sealed

and securely anchored between stronger animal tissues bonded together. Throughout the drying period the lymph was allowed to flow freely through the cannula, but no substantial negative pressure was applied; this was to avoid the risk of the lymph vessel walls collapsing and remaining so, encapsulated in dried tissue adhesive.

Subsequently, the animal was sutured up and the exteriorized tip of the cannula was placed 20-30 cm below animal body level; thus the lymph was drained under slightly negative pressure.

The tubing used for the thoracic duct cannulation was PE 90 (I.D. 0.085 cm. I.D: 0.125 cm) Intramedic Polyethylene tubing, manufactured by Clay Adams Co., U. S. All cannulas were flashed with heparin (1,000 I.U./ml) before placement.

The lymph was collected in polystyrene tubes, containing 0.1 ml Heparin dilution (1,000 I.U./ml) for about 2-4 ml lymph collection. Occasionally, we would mildly agitate the collecting tube to avoid clotting of the collected lymph. When the animals were thrombocytopenic, larger amounts of heparin would be required ($\sim$0.2 ml) to ensure that no clotting would occur in the collected lymph sample.

The surgical procedures and cannulations of the

thoracic duct were performed under an M5 Wild Leitz dissecting microscope and care was taken to ensure that blood vessels we had to cut through were tied up by silk suture both proximal and distal to the point of dissection before partitioning. Thus, we kept bleeding to a minimum, particularly in the abdominal cavity, where no apparent bleeding was visually detected at the time of the post-operative suturing of the animals. Animals in which substantial bleeding into the abdomen had occurred, or in which gross blood contamination of the cannula and lymphatics could be seen under the microscope, or in which the adhesive sealed with tissue did not appear appropriate, were discarded and no measurements were taken.

d. Infusions of anaesthetic, saline, APS, Hydrocortisone, Cr^{51} labelled RBC and drawing of serial blood samples for RBC, platelet counts and radioactivity, was performed via a left carotid indwelling catheter that had its blunted tip placed deep into the aortic arch area. This carotid catheter was made with PE190 Intramedic Polyethylene tubing (I.D.:0.1175 cm, O.D.: 0.1675 cm) manufactured by Clay Adams Co., U.S. An 18 ga needle equipped with a plastic four-way flow control switch was fitted in the external end of this tube.

e. Serial blood samples for CBC and platelet counts

were collected in 3 ml capacity, standard laboratory use, B-D Vacutainers (evacuated glass tubes) manufactured by Becton, Dickinson and Co., Rutherford, New Jersey. The tube interiors were uncoated and the tubes contained 0.06 ml of anticoagulant solution consisting of EDTA 4.5 mg (7.5%), Potassium Sorbate (antimycotic agent) 0.12 mg, and purified water to volume.

Blood samples were taken as follows: via one of the orifices of the cannula valve (the rest of them closed) 1 ml of physiologic saline was injected to clear possible blood clots in the indwelling tip of the cannula. Subsequently 1.5 ml-2 ml of blood were drawn into this plastic syringe; the valve was closed and the first syringe was changed with a second plastic syringe into which 1.5 ml of blood was drawn. The valve was closed, the syringe removed and its contents via an 18 ga needle injected into a vacutainer. Subsequently the blood drawn into the first syringe was reinjected into the animal and washed down with a 1-2 ml of physiological (0.85%) saline. Due to the speed of the operation, no blood coagulation had taken place.

f. Hydrocortisone, used in certain experiments, to test the possible beneficial effect of this drug in

averting the spontaneous extravasation of RBC and subcellular plasma components in thrombocytopenia, was Solu-Cortef (water soluble hydrocortisone sodium succinate) manufactured by the Upjohn Co. We employed the 1 g Mix-O-Vial preparations which when mixed deliver 8 ml solution of the following constitution: hydrocortisone 1 gr, sodium biphosphate (anhydrous) 8.0 mg, dried sodium phosphate 87.3 mg., benzyl alcohol 40 mg, water up to volume.

2. Anti-Platelet Serum

a. Manufacture: Anti-platelet serum (APS). The method used here is basically a slight modification of the techniques developed by earlier investigators (discussed in Chapters 2 and 3, see in particular Rolovic[26], Moore et al[102], Buchanan[103]).

Nine parts of blood are drawn into plastic syringes containing 1 part of 2% EDTA (for details on collections and resuspension of platelets, see Ardlie et al[104] (1970)). The collected blood is centrifuged for 15 minutes at 160 g in room temperature. The supernatant platelet rich plasma (PRP) is collected and centrifuged at 1300 g for 10 minutes in room temperature to a platelet button and platelet poor plasma (PPP). The PPP is discarded and the platelets are washed twice,[102,103,104] with

calcium free Tyrodes buffer (pH 6.2). Platelets in the final button are resuspended in a 2% EDTA solution so that the resultant platelet count in this suspension is at least 5×10^6 platelets/mm^3. The platelets in this preparation are disrupted by sonication and aliquots of the final product are stored at $-20^{\circ}C$ until usage. Thawed aliquots are mixed with equal volumes of Freund's complete adjuvant, emulcified, and injected into guinea pigs; approximate dose, 0.2 ml (0.1 ml sonicated platelet suspension). Half of each injection was given into each side. First injection was into the rear foot-pads, subsequent injections were given subcutaneously at weekly intervals for six weeks. One week after the last injection, the guinea pigs were bled and the prepared serum was heat-inactivated for ~ 60 minutes at $56^{\circ}C$ and denatured protein was removed by centrifugation at 1800 g. The supernatant was filtered through Millipore EAWP 02500, pore size 1.0 μ Celotate filters, split into 5 ml aliquots, stored at lower than $-20^{\circ}C$ and a bioassay was performed in rabbits. In this serum, about 0.3 ml/kg body weight was enough through a single interarterial injection to reduce the platelet counts to less than 10,000 platelets/mm^3 within 10 minutes. Normal serum used in controls was treated in exactly the same manner as the

APS serum.

Sheep anti-rabbit platelet serum with antiendothelial cell activity removed (as manifested by immunofluorescence studies) via absorption with endothelial cells, was similarly manufactured and kindly provided by Dr. J. Gauldie[77, 102]. About twice the amount of this serum was required for the induction of thrombocytopenia levels comparable to the ones induced by the unabsorbed sera.

b. Infusion of APS

This was done via the intracarotid catheter. We usually infused the amount required for severe thrombocytopenia slowly and within 15-30 minutes. The infusion of the serum was followed by an infusion of about 2 ml of physiologic saline. The same procedure was employed for infusion of normal g.p. serum, platelet concentrates and hydrocortisone.

We opted for this method of infusion, instead of the originally tried in our pilot series intravenous administration of APS, due to the fact that the animals tend to breath faster post i.v. infusion of APS, possibly due to the lodging of showers of platelet aggregates in their lungs. This hyperventilation results in a transient increase in lymph flow (see also Ref. 122) which introduces

extra degrees of uncertainty into the system. Furthermore, we wanted to avoid the increased risk of displacing the thoracic duct cannula, due to the rapid breathing movement. For these reasons and because the animals apparently tolerated intra-arterial infusions better, we decided on this approach. The left carotid was consistently utilized throughout these experiments.

3. Platelet Concentrates for Transfusions and Counting of Platelets

a. Platelet concentrates for transfusions. We used Dillard's et al[105] (1951) method with a slight modification; instead of sequestrene solution as anti-coagulant , we used ACD. Six parts of blood are drawn into one part of ACD in a plastic syringe. The collected blood is centrifuged for 15 minutes at 180 g in room temperature for PRP. The supernatant PRP is then centrifuged for 10 minutes at 1300 g in room temperature. Most of the supernatant, PPP, after the centrifugation is discarded, but a small quantity is left in which the platelets are resuspended via gentle agitation in room temperature. Platelet counts of this platelet concentrate varied between $8\text{-}10 \times 10^6$ platelets/mm^3. Platelet concentrates were maintained at room temperature in plastic tubes covered with paraffin film for up to six

hours prior to infusion into thrombocytopenic animals. Time span from the drawing of blood from the donor animals up to the infusion of platelets into the thrombocytopenic animals was about 4-8 hours. These platelet concentrates were mildly agitated before transfusion to ensure uniform suspension. Platelet samples for platelet counts were taken after preparation of concentrate and before transfusion.

b. Platelet counts. They were made by the method of Brecher and Cronkite [106,107] (1950). Blood or platelet concentrates are diluted in 1% ammonium oxalate in distilled water solution; the RBCs are hemolyzed and the platelets are counted in a hemocytometer under a phase contrast microscope. The dilutions were contained in inert plastic tubing (polystyrene) manufactured by Centurus II Ltd., Mississauga, Ontario; transfer of amounts of those dilutions to the hemocytometer was done via siliconized glass Pasteur Pipettes.

4. <u>The Method for Evaluating Red Blood Cells Content in Throacic Duct Lymph</u>

a. The Pilot Series of the experiments. During this part of our work, we tried to establish a fast, reproducible and accurate model for measuring the erythrocyte content of the lymph. In the beginning, we employed electronic cell counters and differential erythrocyte

lysing agents such as zap-isoton. The total cell count/ mm^3 of a given volume of lymph dissolved in physiologic saline would be established via a calibrated model B Coulter Counter[110,111,112] (manufactured by Coulter Electronics Inc., of Florida, U.S.); subsequently, an amount of zap-isoton (manufactured by Coulter Inc.) that would be adequate for complete lysing of RBC was added in the cell suspension and the new cell concentration would be established again in the Coulter Counter. The difference between the first and second counts would yield, when referred to the original volume of lymph used and the final dilution volume, the RBC concentration in lymph. For quality control, we checked the ratio of RBC count in lymph to the total cell count (lymphocytes and RBC) as derived by the method described above, to this ratio as derived by differential cell counting of lymph smears (Wright's Stain, routine Hematology Laboratory[113]). The ratio, as derived by the first method, was consistently higher. Furthermore, the difference between the two ratios and the ratio of the two varied widely from animal to animal, both pre and post-induction of thrombocytopenia; therefore, this was not a question of calibration factors. VanderBoom and Associates[113] reiterated these findings when we ran a series of experiments using their routine hematology

laboratory S Coulter Counter; we simply provided the lymph and they did the rest. Their results verified our findings up to this point.

Consultation with others[109] revealed that they shared our problem experience in differential lysing of mixtures of RBC and lymphocytes. We now attribute this discrepancy, in particular in the post-APS measurement of RBC content of lymph, to the lysing of certain lymphocytes by the used zap-isoton. Possibly, this is due to the cell damaging effect of the required manipulations of lymph and to the cytotoxic effect that anti-platelet sera have on lymphocytes.[102,114]

b. The current model. Eventually, after considering all alternatives, we decided to measure the RBC content in lymph by tagging RBC by the use of appropriate radioisotopes, and by my comparing the irradiation emitted by RBCs in a given volume of lymph to the irradiation emitted by RBCs in a known volume of blood and the known RBC count in blood.

In consultation with Webber et al[115] we decided to utilize ^{51}Cr as the labelling isotope. This isotope has for long been utilized as a reliable RBC tag and is routinely used in blood volume measurements in Nuclear Medicine.[115-120] The added advantage of utilizing this

technique is that we could distinguish whether RBC in lymph were the result of direct bleeding into the abdominal cavity and their subsequent pick-up by the lymphatics, or of spontaneously extravasated RBCs. This distinction could be accomplished due to the fact that infusion of labelled RBCs into the animals was performed usually 2-3 hours post-operatively, at which time any possible bleeding would have been arrested; thus, since any blood cells already extravasated were not tagged, only spontaneously extravasated labelled cells would be found in lymph.

The RBC count in lymph is measured by this technique (see below for technical details) compared very well to the RBC concentrations in lymph as derived by multiplying RBCs percentage content in lymph, estimated from differential reading of lymph smears, by the total cell count in lymph as counted via the Coulter Counter. Allowing for estimated probable errors (due to measuring of volumes of lymph and blood, calibration errors of the Coulter Counter, and inherent statistical errors of: i. Coulter Counter measurements; ii. differential smear counts; and iii. irradiation emission counts) and assuming a Poisson distribution, we found that the RBC counts in lymph as calculated by the two methods, from

split samples of homogeneous lymph suspensions, were variates (within 90% confidence limits)[121] of measuring the same mean (true) count.[121] Only occasionally (and within statistical chance expectations for such deviant measurements) would the 90% confidence limit be exceeded. This was due possibly to either statistical chance or an underestimation of probable errors for these particular measurements.

5. Labelling of RBCs with $Na_2{}^{51}CrO_4$

Labelling of RBCs with ^{51}Cr. This was done by Webber and Associates in the Nuclear Medicine Laboratories at MUMC as follows (we give quantities utilized/rabbit, in order to achieve sufficient lymph RBC radioactivity levels): i. 7-10 ml of blood are drawn into a plastic syringe containing ACD (6 parts blood to 1 part ACD); ii. the blood is centrifuged and the RBCs layer is separated from the rest of the cellular elements and PRP; iii. approximately 120 μCi of $Na_2{}^{51}CrO_4$ solution (usually 1 mCi/ml) is added to the packed RBCs and the system is incubated in room temperature for 30 minutes under gentle non-continuous agitation. During this state $++CrO_4$ ions cross the RBC cell membrane and get reduced to Cromic ions, which cannot cross RBC membranes.[116,117] These cromic ions get firmly attached to the globin portion

of the hemoglobin molecule and there is no exchange of ^{51}Cr with plasma, except for elution. Outside the red cells, most of the plasma chromium is bound to globulin and albumin;[117] iv. the RBCs are washed twice with saline to remove any traces of unabsorbed ^{51}Cr, as follows: a. add saline to make up original volume, leave a couple of minutes (agitate once), centrifuge; b. repeat stage a; v. saline is added up to the original volume; vi. infuse into recipient animal. We must add here that when ^{51}Cr labelled RBCs are lysed in vivo, the freed radioactivity does not tag other red cells. Ebaugh et al[120] (1953) were able to demonstrate that the injection of chromated hemoglobin or of ^{51}Cr labelled non-viable cells is followed by a rapid fall of circulating radioactivity to zero.

6. Preparation of Lymph Samples and of Blood Samples for Radioactivity Counting

a. Lymph. The collected heparinized lymph samples were agitated to ensure uniform distribution of cells and a 1 ml aliquot was drawn into 1 ml plastic syringe. This was placed into a polystyrene plastic tube and 2 ml of physiologic saline was added. The addition of the saline was an added precaution and aimed at minimizing the amount of free radioactivity that would remain in the cell button which is received by centrifugation of

this dilution in room temperature and at 1300 g for 15 minutes. The supernatant was removed via a siliconized Pasteur pipette and placed into a gamma counting vial (manufactured by Amersham Searle Co.). In the button, consisting of a bottom layer of labelled RBCs and a layer of lymphocytes in the top, we added 1.5 ml of physiologic saline and zap-isoton to lyse the RBCs. This mixture was agitated to ensure an homogeneous radioactive volume for more accurate radioactivity measurements. The polystyrene tube containing this mixture was placed into another gamma counting vial. The tubes were serially numbered and radioactivity measurements were made in an automatic well-type gamma counter (Nuclear Chicago, now Searle).

b. Blood. Blood samples post-infusion of labelled RBCs were drawn after at least 60 minutes post-infusion This time period is most adequate for homogeneous distribution of the labelled RBC within the total RBC population to take place.[118] Serial samples were taken at later times. From each of those samples, 44.7 mm^3 of blood was drawn into a standard hematology laboratory graded pipette and transferred into 10 ml of physiologic saline, contained in a plastic container. This dilution was mildly agitated to a homogeneous cell suspension and

1 ml of this suspension was drawn and treated exactly the same as the 1 ml of the lymph sample, discussed above. The final preparations, contained in the gamma counting vials, were placed in the same well-type gamma counter as the lymph samples were. In the above discussed technique, the influence of free plasma radioactivity on the blood RBC radioactivity measurements has been estimated at less than $1:10^4$. For the lymph samples, the equivalent figure is about 1:100. These estimates were calculated on the basis of the experimental values, dilution ratios, and on the assumption that after centrifugation, the free radioactivity in the supernatant was uniformly distributed.

7. The Numerical Calculations for RBC Counts in Lymph

As we discussed previously in the preparation of samples for the radioactivity measurements in blood, 44.7 mm^3 of blood were diluted in 10 ml of physiological saline and 1 ml of this dilution was centrifuged; thus, the obtained RBC button contained as many RBCs as 4.7 mm^3 of whole blood. Let us assume that the actual (measured minus background) radioactivity for these RBCs is (BR) and let us also assume that the RBC count/mm^3 in blood is (BC).

Since 1000 mm^3 of lymph was centrifuged, there will be as many RBCs in the final button as is contained in 1000 mm^3 of lymph. Radioactivity measurements (emission counts) for this specimen, expanded over the same period of time as for the blood sample above, yield an actual numerical value that we assume to be (LR). The RBC count/mm^3 in lymph is the unknown quantity (LC).

If the statistical average radioactivity per RBC is R (this average value has real meaning only if large enough number of RBCs are sampled) then:

$$R = \frac{(BR)}{(BC) \times 4.47} = \frac{(LR)}{(LC) \times 10^3}$$

From the above equation, we derive the value of (LC) as follows:

$$(LC) = \frac{(BC) \times 4.47}{(BR) \times 10^3} \times (LR)$$

or $(LC) = A \times (LR)$

where A is a constant, unless substantial numbers of new unlabelled RBCs come into the circulation and/or large numbers of the labelled group cells are destroyed preferentially.

8. <u>RBC Flux in Lymph Vessels</u>

We define the number of red cells contained in the

volume of lymph that flows through a cross section of the thoracic duct in one hour, as the red cell flux through the duct, (at this cross section). This quantity equals the average RBC concentration per unit volume in a particular sample of lymph, multiplied by the hourly flow of lymph expressed in the same volume units and for the same sample.

The red cells flux may be a more accurate reflection of the degree of spontaneous escape of RBCs from the vascular compartment than the RBC concentration in lymph. The latter is more susceptible to variations in response to fluctuations in the amount of fluid circulated through the lymphatics for a given time period. However, in the presentation of our results, we discuss both the RBC concentration in thoracic duct lymph and the RBC flux through the duct.

CHAPTER 6

EXPERIMENTS AND RESULTS: PART A

1. Experimental Procedures for Controls and for APS Treated Animals

We usually allowed for about two and one half hours past the thoracic duct cannulation operation before transfusion of the ^{51}Cr tagged homologous RBC.

The lymph collecting tubes were changed every one half to one hour (or sooner when fast flowing lymph warranted doing so), and the volume of the collected lymph was measured and aliquot samples of 1 ml of lymph were drawn from the collecting tubes, for testing for lymph RBC content.

The labelled RBC concentration in lymph usually reached a plateau within one and one half to two hours post transfusion and fluctuated around this plateau value for the remainder of the experiments, unless severe thrombocytopenia was induced. We ran a number of animals (5) up to ten hours post-transfusion of labelled RBCs without any significant deviation from the pattern described above. Another five animals were used to define plateau values up to six hours post-transfusion of tagged red cells. Four animals were infused 1.5 ml of normal

g. p. serum which exceeds the maximum volume of g. p. anti-rabbit platelet serum required for the induction of thrombocytopenia in this experiment. This infusion was performed four hours post-transfusion of tagged RBCs and the animals were followed for up to four hours post-transfusion of the serum without any significant changes being observed in lymph RBC content.

For individual experiments, the animals were used as their own controls to establish their plateau RBC concentration levels in lymph before the induction of thrombocytopenia. We usually allowed for about four hours post-transfusion of labelled RBCs before treatment of the animals with APS. Post APS treatment and the induction of thrombocytopenia, lymph samples were taken every one half to 1 hour for up to four hours. Blood samples were drawn throughout the experiment and the minimum number of samples was three, one at one-half hour pre-infusion of APS, and the other two at one half hour and two to three hours post-infusion of APS. Blood samples were used for establishing RBC and platelet counts and for measuring red cell bound radioactivity levels.

2. Baseline Measurements

The following baseline measurements refer to the anaesthetized non-thrombocytopenic rabbits.

i. RBC Concentration in Thoracic Duct Lymph

The mean concentration was found to be 760 RBC/mm^3 of lymph, with a standard deviation of 809 RBC/mm^3 and with a minimum of 77 RBC/mm^3 and a maximum of 2800 RBC/mm^3. The distribution is skewed with 69% of the animals having lymph RBC concentrations below the mean. The median of the distribution is at about 400 RBC/mm^3 (50% of animals have RBC concentration in lymph below this value and 50% above this value) and the pick of the frequency distribution was for the 0-250 RBC/mm^3 group with about one third of the animals included into this category.

ii. Thoracic Duct Lymph Flow

The mean hourly lymph flow was 4.8 ml, with a standard deviation of 1.6 ml/hr and a minimum of 1.9 ml/hr and a maximum of 9.6 ml/hr. The median of this distribution was 4.5 ml (close to the mean value) and the distribution is almost a bell shape one.

iii. RBC Flux in thoracic Duct Lymph

The mean RBC flux was found to be 3.4×10^6 RBC/hr with a standard deviation of 3.6×10^6 RBC/hr and a minimum of 0.17×10^6 RBC/hr and a maximum of 17.0×10^6 RBC/hr. This distribution is also skewed with 69% of the animals having a RBC flux lower than the mean.

The median is at about 2.2×10^6 RBC/hr and the pick of the frequency distribution is for the $1.0\text{-}2.0 \times 10^6$ RBC/hr group with 26% of the animals having RBC lymph fluxes within this range.

3. The Anti-Platelet Serum Infused Animals

Twenty-two rabbits were rendered severely thrombocytopenic by intra-arterial administration of anti-platelet serum. Fourteen of these animals were treated with guinea pig anti-rabbit platelet serum and eight with sheep anti-rabbit platelet serum. The latter had its anti-endothelial cell activity removed (as manifested with immunofluorescence studies) by incubation with endothelial cells[77]. In both groups, thrombocytopenia levels of less than 10,000 platelets/mm^3 were established within one half hour post-infusion (see also Refs. 46 and 54). No RBC lysis could be detected, as judged either from the coloration of the supernatant of the centrifuged blood samples before and post-APS, or from studying the ratio:

$$K = \frac{\text{blood plasma radioactivity}}{\text{RBC bound radioactivity in blood.}}$$

We hoped that lysis of RBCs would be reflected in an increase of K values. However, if indeed lysis occurred, the lysed radioactive debris of RBCs must have been removed from circulation as early as one-half hour post

APS, since blood samples taken as early as that did not reveal an increase in K values. On the contrary, a decrease in the value of K, from an average of 1.9% down by about 20%, was detected. We attributed this decrease to the possibility that, with the onset of thrombocytopenia, the increase in vascular permeability for subcellular plasma components outweighs the increase in vascular permeability for erythrocytes. Thus, radioactivity bearing plasma components moved faster out of circulation than radioactivity bearing RBCs.

There was a transitory change in blood RBC concentrations post APS. This tended to be compensated later on. The RBC concentration in blood was decreased by an average of 1% for the guinea pig anti-rabbit platelet serum treated animals, and for an average of 2.3% for the free from anti-endothelial cell activity serum treated animals.

4. RBC Concentration in the Thoracic Duct Lymph of Thrombocytopenic Animals

In the discussion below, we designate the animals treated with the regular anti-platelet serum as the T group (14 animals) and the animals treated with the endothelial cells absorbed anti-platelet serum as the E group (8 animals).

For nine of the animals in group T and six of the animals in group E, increased RBC concentrations in lymph were measured in samples collected one-half hour post-infusion of APS. The rest of the animals, except E8, had increased levels of RBC concentrations in lymph within one hour post-infusion of the sera. Animal E8, which had the highest post-APS platelet count of about 10,000 platelets/mm^3, did not have its RBC concentration in lymph increased for the duration of the experiment.

For eleven animals in the T group and for six animals in the E group, an apparent plateau of lymph RBC concentration was reached, within four hours post-APS. The remainder of the animals had increasing levels of concentrations of RBCs in lymph for the duration of the experiments.

For ten of the animals in the T group and for five of the animals in the E group, the final measurement of lymph RBC count was lower than the maximum value. In Table 6.4.1, we give: i. the mean and standard deviation for RBC concentration in lymph, as measured by a multitude of serial samples before administration of APS; ii. the maximum measured value of RBC concentration in lymph post-APS; iii. the last measured value; iv. the post-APS platelet count; v. the d values and levels

of statistical significance, by which we reject the hypothesis that the maximum measured RBC concentration in lymph post-APS is a random measurement of the measured RBC concentration in lymph values, as they vary with time in normal rabbits. We are assuming the pre-APS measured values to have a normal distribution (we are referring to a multitude of serial measurements in the same animal); vi. the levels of statistical significance for rejecting the hypothesis that the maximum measured RBC concentration in lymph value and the last recorded value are random measurements for the same RBC concentration. For this test, we assume that those variate measurements of the same (true) value follow a Poisson distribution; vii. in this column we give levels of significance as above, but assuming an estimated error of 10% for these measurements.

The obvious results demonstrated in Table 6.4.1 are:

a) There is a statistically significant increase in RBC concentration in lymph post-APS when severe thrombocytopenia is induced.

b) Within four hours post-APS, in a substantial number of animals, there is a tendency for a partial decrease of the elevated RBC concentration in lymph.

Therefore, it would appear that maximum RBC concentration levels have been reached within four hours post-APS and the induction of severe thrombocytopenia.

We next tested the hypothesis that animals infused with the endothelial cells absorbed APS and with such doses as to develop comparable levels of thrombocytopenia with animals treated with regular APS, would have maximum lymph RBC concentration values different than those reached in the second group.

To test this hypothesis, we did the Gosset t test for the following three pairs of distributions:

i. Mean RBC concentration in lymph before treatment with APS, T and E groups.

Result: $t_{20} = 0.21$;

ii. Maximum RBC concentration in lymph post-treatment with APS, T and E groups. Entire T population.

Result: $t_{20} = 1.22$;

iii. Maximum RBC concentration in lymph post-treatment with APS, E group animals matched to number of T group for same platelet levels post-APS.

Result: $t_{14} = 0.01$.

The above t test values demonstrate that there is not statistically significant difference in the effect

of these two sera in elevating the RBC counts in lymph, (as judged by maximum RBC counts), for comparable levels of thrombocytopenia.

5. Thoracic Duct Lymph Flow Rates in Thrombocytopenic Animals

A multitude of factors such as breathing rate, other movements of the animal and degree of anaesthesia bring about rapid transient changes in lymph flow rates. In these experiments, such changes in lymph flow rates were often observed post-APS infusion, quite commonly in association with some apparent transient acceleration of breathing rate. However, those changes were transient and when averaged over the post-APS period of the experiments, tended to be minor. The average increase from the pre-APS flow rates was about 22%. There was also some compensation in the sense that prolonged faster lymph flow rates tended to be followed by a slow down at below pre-APS levels. On a few occasions, we also had a slow down of lymph flow post-APS.

In Table 6.5.1, we present the lymph flow rates data for both the T and E groups as follows: i. mean and standard deviation pre-APS; ii. mean and standard deviation post-APS; iii. platelet count post-APS.

The t test for the distribution of the mean

values pre-APS and post APS and for the whole group (T and E) of animals gives a t_{42} value of 1.94. This is just under the 2.021 t_{40} value for a level of 5% statistical significance level.

6. RBC Flux in the Thoracic Duct of Thrombocytopenic Animals

The maximum RBC concentration in lymph post-APS and the maximum lymph flow rates do not always coincide, as there is usually some tendency for higher lymph flow rates shortly post-infusion of APS, whereas it takes some time for RBC concentration in lymph to reach maximum values. Thus, it is possible for a lymph flow rate higher than the lymph flow rate for the maximum RBC concentration in lymph sample to yield, in multiplication with the RBC concentration for the same sample, a RBC flux value higher than the one corresponding to the maximum RBC concentration sample. Lymph flow rates higher than the one corresponding to the maximum RBC concentration in lymph sample do occur both pre and post this sample.

The above considerations are amply demonstrated in animals T1 and E3 (see Tables 6.4.1. and 6.6.1). In animal T1, the last RBC concentration measurement becomes the peak of the RBC flux values distribution because of

a slight increase of lymph flow rate that is faster than the lymph flow rate corresponding to the maximum RBC concentration in lymph sample. However, neither of these flow rates was the highest for this experiment. The reverse situation is depicted in animal E3, where a slow lymph flow rate at the end of the experiment resulted in a high RBC concentration in lymph, when in reality, faster lymph flow rates at earlier samples resulted in much higher RBC flux values for previous measurements. However, in a substantial number of animals, high lymph flow rates did coincide with high RBC concentration in lymph values, and therefore in the majority of the animals, the maximum RBC concentration in lymph measurement and the maximum RBC flux in lymph measurement, coincided.

We next proceed to evaluate our data for the RBC flux values in lymph for the groups of animals T and E, using the same approach we used in evaluating our RBC concentration in lymph data.

In the columns of Table 6.6.1, we give: i. the mean and standard deviation for the RBC flux in thoracic duct lymph, as measured for each animal by a multitude of serial samples before infusion of APS; ii. the maximum measured such value post-APS; iii. the

last measured such value for this experiment; iv. the post-APS platelet count; v. the level of statistical significance at which we reject the hypothesis that the maximum measured, post-APS, RBC flux in t. d. lymph is a random measurement of the measured RBC fluxes pre-APS, and for the same animal. We are assuming, as we did for the RBC concentration values, that those values follow a normal distribution (we are referring to a multitude of serial measurements in the same animal and not to the distribution of the means of these values for the entire population). In this column, the d values for such a significance test and the level of statistical significance are given; vi. the level of statistical significance for rejecting the hypothesis that the measured maximum RBC flux and the last RBC flux measurement are random measurements of the same RBC flux level. For this test, we assume a Poisson distribution; vii. in this column, we give the levels of significance as above, but assuming an estimated error of 10% for these measurements.

There are no new conclusions to draw from this table, other than the ones already discussed in Section 6.4, for the RBC concentration data in thoracic duct lymph.

We now proceed to test the hypothesis that animals infused with the endothelial cell absorbed serum (E group) will develop different levels of maximum RBC fluxes in thoracic duct lymph, post-APS, than animals treated with regular APS and for comparable levels of thrombocytopenia.

As we did before in Section 6.4, we tested here the following three pairs of distributions:

i. Mean RBC flux in lymph before APS

Groups T and E

Results: $t_{20} = 0.789$

ii. Maximum RBC flux values in t. d. lymph

Post-administration of APS, T and E

Entire T population

Results: $t_{20} = 0.92$

iii. Maximum RBC flux values in lymph post-APS

E group animals matched to numbers of T group for same level of platelet counts post-APS

Results: $t_{14} = 0.51$.

These t test values demonstrate that there is no statistically significant difference between the two groups on the basis of their maximum RBC fluxes in lymph post-APS.

Of interest is the observation that RBC flux in

lymph and RBC concentration in lymph data indicate that these values reach a first maximum within four hours post-APS, although in a number of animals continued to rise past the four-hour period.

TABLE 6.4.1.

RBC concentration in the thoracic duct lymph of thrombocytopenic animals.

ANIMAL	RBC concentration in thoracic duct lymph. x 1,000/mm^3. Pre-APS Mean & s.d.	Post-APS. Max.	Post-APS. Last.	Platelet level. Post-APS. x 10^3/mm^3.	d value & statistical significance RBC:pre-APS, vs,max post-APS.	Statistical significance RBC:max,vs, last.Both post-APS.	
T 1	0.54 ; 0.23	1.45	1.39	10.	4.1 ; 0.1%	----	----
T 2	0.32 ; 0.05	3.94	0.89	7.	80. ; 0.1%	0.1%	0.1%
T 3	1.28 ; 0.31	4.31	4.31	4.	9.8 ; 0.1%	----	----
T 4	0.85 ; 0.09	5.40	4.75	5.	53. ; 0.1%	0.1%	----
T 5	0.14 ; 0.05	3.91	2.57	8.	82. ; 0.1%	0.1%	0.1%
T 6	1.49 ; 0.36	5.31	3.49	4.	10.6 ; 0.1%	0.1%	0.1%
T 7	0.54 ; 0.39	4.99	4.77	6.	11.2 ; 0.1%	0.1%	----
T 8	0.74 ; 0.33	3.69	3.69	4.	8.7 ; 0.1%	----	----
T 9	0.16 ; 0.02	81.21	81.21	1.	99+ ; 0.1%	----	----
T10	2.88 ; 0.52	28.54	15.89	4.	40. ; 0.1%	0.1%	0.1%
T11	0.60 ; 0.17	25.28	25.28	6.	99+ ; 0.1%	----	----
T12	2.67 ; 0.40	24.99	14.59	3.	55. ; 0.1%	0.1%	0.1%
T13	0.20 ; 0.04	10.10	5.97	2.	99+ ; 0.1%	0.1%	0.1%
T14	0.26 ; 0.04	20.52	12.97	3.	99+ ; 0.1%	0.1%	0.1%
E 1	2.37 ; 0.43	3.59	3.59	7.	2.8 ; 1.0%	----	----
E 2	0.15 ; 0.09	1.09	0.55	6.	10. ; 0.1%	0.1%	0.1%
E 3	0.23 ; 0.04	2.20	2.20	8.	54. ; 0.1%	----	----
E 4	0.99 ; 0.18	14.17	7.27	6.	74. ; 0.1%	0.1%	0.1%
E 5	2.76 ; 0.47	3.73	3.54	4.	2. ; 5.0%	1.0%	----
E 6	0.17 ; 0.07	5.73	2.50	4.	83. ; 0.1%	0.1%	0.1%
E 7	0.28 ; 0.06	20.64	14.70	6.	99+ ; 0.1%	0.1%	0.1%
E 8	1.10 ; 0.25	0.50	0.50	10.	2.4 ; 5.0%	----	----

TABLE 6.5.1.
Thoracic duct lymph flow rates in thrombocytopenic animals.

ANIMAL	Thoracic duct lymph flow rates. x 1ml/hr. Pre-APS. Mean & s.d.	Post-APS. Mean & s.d.	Platelet level. Post-APS. x $10^3/mm^3$.
T 1	6.3 ; 1.45	5.5 ; 1.05	10.
T 2	4.6 ; 0.28	4.3 ; 0.99	7.
T 3	4.2 ; 0.93	4.7 ; 0.66	4.
T 4	6.4 ; 0.51	4.8 ; 0.82	5.
T 5	3.9 ; 0.08	4.5 ; 0.90	8.
T 6	4.5 ; 0.68	5.2 ; 1.14	4.
T 7	6.8 ; 1.77	7.0 ; 1.96	6.
T 8	3.7 ; 1.07	5.2 ; 2.50	4.
T 9	6.6 ; 1.58	7.4 ; 1.26	1.
T10	4.0 ; 0.68	5.1 ; 1.02	4.
T11	4.6 ; 0.55	6.2 ; 0.43	4.
T12	3.1 ; 0.71	3.5 ; 0.74	3.
T13	4.5 ; 0.50	7.3 ; 0.65	2.
T14	6.7 ; 1.27	10.2 ; 1.84	3.
E 1	4.0 ; 0.08	8.3 ; 1.25	7.
E 2	2.8 ; 0.28	2.5 ; 0.50	6.
E 3	9.6 ; 0.58	10.6 ; 3.29	8.
E 4	7.8 ; 0.23	7.4 ; 1.33	6.
E 5	6.3 ; 0.57	10.2 ; 2.35	4.
E 6	7.3 ; 1.83	7.1 ; 3.05	4.
E 7	4.1 ; 0.90	7.2 ; 1.73	6.
E 8	3.4 ; 0.58	6.7 ; 1.74	10.

TABLE 6.6.1.
RBC flux in the thoracic duct lymph of thrombocytopenic animals.

ANIMAL	RBC flux in thoracic duct lymph. x 10^6/hr. Pre-APS. Mean & % s.d.	Post-APS. Max.	Post-APS. Last.	Platelet level. Post-APS. x $10^3/mm^3$.	d value & statistical significance RBC flux: pre-APS, vs, max post-APS.	Statistical significance RBC flux: max, vs, last. Both post-APS.	
T 1	3.24 ; 1.46	8.34	8.34	10.	3.4 ; 0.1%	----	----
T 2	1.50 ; 0.23	18.91	3.29	7.	77. ; 0.1%	0.1%	0.1%
T 3	5.60 ; 1.62	21.12	21.12	4.	9.5 ; 0.1%	----	----
T 4	5.51 ; 0.44	24.30	22.90	5.	42. ; 0.1%	0.1%	----
T 5	0.56 ; 0.18	17.60	12.85	8.	95. ; 0.1%	0.1%	0.1%
T 6	6.59 ; 1.38	29.21	12.92	4.	16. ; 0.1%	0.1%	0.1%
T 7	3.37 ; 1.52	39.87	24.79	6.	24. ; 0.1%	0.1%	0.1%
T 8	2.52 ; 0.43	18.07	18.07	4.	36. ; 0.1%	----	----
T 9	1.06 ; 0.30	503.50	503.50	1.	99+ ; 0.1%	----	----
T10	11.17 ; 2.23	148.41	61.97	4.	61. ; 0.1%	0.1%	0.1%
T11	2.75 ; 1.16	146.62	146.62	6.	99+ ; 0.1%	----	----
T12	8.12 ; 1.46	94.97	37.94	3.	57. ; 0.1%	0.1%	0.1%
T13	1.00 ; 0.23	69.72	42.36	2.	99+ ; 0.1%	0.1%	0.1%
T14	1.68 ; 0.13	229.87	102.43	3.	99+ ; 0.1%	0.1%	0.1%
E 1	9.50 ; 1.71	26.19	26.19	7.	9.7 ; 0.1%	----	----
E 2	0.45 ; 0.32	2.85	1.38	6.	7.3 ; 0.1%	0.1%	0.1%
E 3	2.16 ; 0.39	27.30	14.74	8.	65. ; 0.1%	0.1%	0.1%
E 4	7.76 ; 1.24	110.53	46.53	6.	83. ; 0.1%	0.1%	0.1%
E 5	17.38 ; 2.61	45.81	25.84	4.	11.6 ; 0.1%	0.1%	0.1%
E 6	1.20 ; 0.41	39.54	11.11	4.	94. ; 0.1%	0.1%	0.1%
E 7	1.14 ; 0.36	132.10	77.91	6.	99+ ; 0.1%	0.1%	0.1%
E 8	4.08 ; 0.86	2.82	2.82	10.	1.4 ; ----	----	----

CHAPTER 7

EXPERIMENTS AND RESULTS: PART B

In this chapter, we describe two pilot series of experiments as an application of the present experimental model and the results discussed in the previous two chapters.

1. The Hydrocortisone Experiments

Four animals (group H) were infused intra-arterially with Hydrocortisone as follows. Three animals were given a bolus injection of about 0.1 gr/kg body weight one-half hour pre-APS. The fourth animal (H4) was given 1 gr Hydrocortisone (animal weight, 2.9 kgr) in four equal bolus injections at one-half hour intervals, the last injection at one-half hour pre-APS. In all other aspects, these experiments were conducted as the ones described inthe previous chapter. In Table 7.1.1, we give the RBC concentration in lymph measurements for these animals as follows: i. mean and standard deviation, RBC concentration in lymph pre-Hydrocortisone and pre-APS treatment; ii. maximum RBC concentration in thoracic duct lymph post-Hydrocortizone and post-APS treatments; iii. last measured RBC concentration in

thoracic duct lymph; iv. platelet count post-APS.

In Table 7.1.2., we give the data for RBC flux in lymph measurements in the same order as for the RBC concentration in lymph measurements in Table 7.1.1.

The post-APS platelet level range for these results is 2-7,000 platelets per cubic milimeter blood. We compared the results of group H with the results for groups T and E and for the animals in the same post-APS platelet level range.

The t test values for the maximum post-APS RBC concentration values in the two groups of animals was found to be: $(H)-(T-E)_{2-7}t_{19} = 2.01$, which is statistically significant at the 10% level and almost at the 5% (2.093).

The t test values for the maximum post-APS RBC flux measurement in the lymph of the animals of the two groups was found to be: $(H)-(T-E)_{2-7}t_{19} = 0.119$, no statistically significant difference.

The above discrepancy is due to the apparently slower post-APS lymph flow rates in the Hydrocortisone pretreated thrombocytopenic animals. Indeed, for this group of animals, the average hourly lymph output, for the post-APS period, was reduced by an average

of 2.5% in respect with their pre-APS average lymph flow rates. Since in the thrombocytopenic animals in groups T-E the average increase in lymph flow rates post-APS was 22%, it would appear that the RBC-measurements in the lymph of animals in group H are more concentrated than for animals in groups T-E. However, the products of the higher RBC concentrations times the lower lymph flow rates (e. g., the RBC flux) did not seem to be strongly affected, and there was no statistically significant difference between group H and group T-E.

In summary, the above study indicates the need, when one studies the extravasation of erythrocytes from the vascular compartment via sampling of lymph, to utilize the RBC flux in lymph criterion, rather than the RBC concentration in lymph measurements. Furthermore, the above preliminary study indicates that (for doses utilized here) whereas hydrocortisone does not significantly affect the number of extravasated erythrocytes in thrombocytopenia, it may be reducing the fluid return rate into the vascular compartment via the lymphatics.

2. The Fresh Platelet Concentrates Transfusion Experiments

We next proceeded to investigate the efficacy of fresh platelets in plasma concentrate transfusions in raising the platelet counts of anti-platelet serum

rendered thrombocytopenic animals.

A group of five animals (group P) was operated on and rendered thrombocytopenic in the mode described in Chapters 5 and 6. At two hours post-APS, the animals were transfused slowly with platelet concentrates (within 15 minutes). In Table 7.2.1, we give the results for this group of animals as follows: 1. pre-platelet transfusion platelet count; ii. post-platelet transfusion expected platelet count (assuming a blood volume in lt approximately equal to 7% body weight in kg); iii. post-platelet transfusion actual platelet count at one hour post-transfusion; iv. percentage: actual/expected platelet counts post-transfusion; v. RBC concentration in lymph before transfusion of platelets; vi. RBC concentration in lymph two hours post-transfusion of platelets; vii. RBC flux in lymph pre-transfusion of platelets; viii. RBC flux in lymph two hour post-transfusion of platelets.

We compared the final measurements for RBC concentration in lymph and for RBC flux in lymph in this group with those of the animals in group T-E with post-APS platelet counts in the same range as for group P. The t test values for the RBC concentration and for the RBC fluxes in lymph are correspondingly (RBC concentration)

t_{20} = 1.94 and (RBC flux) t_{20} = 1.98. Statistical significance for both is at the 10% level, and the final measurements are lower for group P than for group T-E.

However, what is pertinent to this work is not this marginal statistically significant difference, but the fact that platelets can be transfused and survive into anti-platelet serum rendered thrombocytopenic animals, shortly after the administration of the antiserum.

TABLE 7.1.1

The effect of Hydrocortisone on the RBC concentration in the lymph of thrombocytopenic animals.

ANIMAL	RBC concentration in thoracic duct lymph. x 1,000/mm^3. Pre-APS Mean & s.d.	Post-APS. Max.	Post-APS. Last.	Platelet level. Post-APS. x 10^3/mm^3.
H 1	0.36 ; 0.21	8.53	8.53	2.
H 2	2.66 ; 0.72	29.78	27.50	4.
H 3	0.83 ; 0.32	15.11	14.00	5.
H 4	1.16 ; 0.24	26.73	26.54	7.

TABLE 7.1.2

The effect of Hydrocortisone on the RBC flux in the lymph of thrombocytopenic animals.

ANIMAL	RBC flux in thoracic duct lymph. x 10^6/hr. Pre-APS. Moan & s.d.	Post-APS. Max.	Post-APS. Last.	Platelet level. Post-APS. x 10^3/mm^3.
H 1	2.26 ; 0.77	28.15	21.33	2.
H 2	9.88 ; 2.47	107.21	90.75	4.
H 3	2.40 ; 0.53	33.24	30.80	5.
H 4	2.96 ; 0.38	128.30	84.91	7.

TABLE 7.2.1.

The Effect of fresh platelets transfusions on the RBC concentration in the lymph of thrombocytopenic animals.

ANIMAL	Platelet level. Post-APS. x 1,000/mm^3. Pre-transfusion.	Post-transfusion of fresh platelets. Expected.	Actual.	%	RBC concentration in thoracic duct lymph. x1,000/mm^3. 2 hrs post-APS.	2 hrs post-transf.	RBC flux in thoracic duct lymph. x10^6/hr. 2 hrs post-APS.	2 hrs post-transf.
P1	6.	274.	136.	51%	2.86	0.51	16.32	2.31
P2	7.	219.	89.	38%	4.46	1.51	15.61	5.27
P3	3.	280.	115.	40%	2.08	1.44	9.16	4.75
P4	7.	259.	119.	46%	1.87	2.97	7.31	9.20
P5	4.	86.	21.	24%	6.64	1.57	34.54	7.39

CHAPTER 8

DISCUSSION AND SUMMARY

1. Applicability of the APS Thrombocytopenic Model

Qualitative[82] electron microscopic studies and quantitative[10,18,20-23] lymph sampling studies have shown that in irradiation induced experimental thrombocytopenia, there exists an increased rate of extravasation of erythrocytes from the intact blood vessels. However, in the anti-platelet serum experimental thrombocytopenic model, our extensive review of the literature yielded only qualitative electron microscopic studies of the phenomenon.[53,54]

We have now demonstrated quantitative increases of RBC concentration values in lymph, lymph flow rates and RBC flux in thoracic duct lymph in APS induced thrombocytopenia. The initial rise of RBC concentrations in lymph is followed in the majority of the animals by a later decrease, but still remains well above pre-APS levels. Also, the initial outpouring of lymph tends to be followed by a later slow down, even below pre-APS levels.

We have also demonstrated that fresh platelets can be transfused into the thrombocytopenic animals

shortly post-administration of APS, thus making this model a reversible thrombocytopenic model appropriate for the study of the anti-purpuric efficacy of platelets stored in different temperatures and/or in different suspension media. The model also appears to be sensitive to the effect of pharmaceuticals on the permeability of the vascular wall to cellular and subcellular plasma components in thrombocytopenia. Thus, it renders itself to the study of compounds such as corticosteroids, long supposed as having anti-purpuric properties.

The reproducibility of this model and the fact that the animals can be operated while they are not thrombocytopenic, and that the whole experiment can be performed in one day are some of the considerations in favour of it. Furthermore, the fact that the infusion of tagged RBC takes place post-operatively reduces the possibility that direct bleeding in the abdominal cavity during the operation may contribute to an artificial increase of RBC counts in lymph, as some of these RBC find their way back into the circulation via the lymphatics.

2. Comparison of Results in the Irradiation and in the APS Thrombocytopenic Models

Of interest is a comparison between the extent of thrombocytopenic erythrocyte extravasation and increased

lymph RBC content in our experiments, and those of Woods et al[18], Jackson et al[20], Roy et al[21] and Aursness.[22,23]

Our results are in line with those of Aursness, whereas the results of Woods et al, Jackson et al, and Roy et al, indicate much higher levels of erythrocyte extravasation. We postulate that the difference is due perhaps to lymph node tissue degeneration and direct hemorrhagic tendencies in the lymph nodes of the irradiated thrombocytopenic animals.[8,10,11] Aursness' results of lower RBC counts in lymph may therefore be due to the fact that he cannulated lymph vessels that drained tissues with only type I capillaries, and before these vessels enter any lymph nodes, thus minimizing the chance for a lymph node direct bleeding component. We utilized a thrombocytopenic model for which we do not have reason to presume a lymph node damaging effect of the extent manifested in post-irradiation thrombocytopenia via histologic studies;[8] however, we drained lymph derived from tissues with type I capillaries (muscle), type II capillaries (intestinal mucosa) and type III capillaries (bone marrow). We postulate that had we cannulated lymph vessel instead, draining only tissues with type I capillaries, we would have found RBC concentrations in

lymph to be lower than those of our current work and those observed by Aursness.

Another possible contributing factor accounting in part for the much higher RBC concentrations in lymph observed by other investigators may be that in most of their work, they cannulated the thoracic duct cephalad to our point of cannulation, in the supraclavicullar region, thus draining lymph that was partially derived from the liver and spleen lymphatic drainage system, both the liver and spleen being organs with type III capillaries and massive blood supply. Since those capillaries are permeable to RBC even in normal animals, it may well be that their permeability to RBC in thrombocytopenia is increased greatly compared to the increase for type I and type II capillaries. Also, species differences do exist and may have contributed to the observed differences in RBC concentration in lymph of the thrombocytopenic animals; only Bigelow et al and Aursness reported on the rabbit in their experiments (as we did).

We feel, however, that it is not really all that meaningful to attempt numerical comparisons in measurements of RBC concentrations in lymph between the irradiation

thrombocytopenia and the APS thrombocytopenia for the following possible reasons: i. if indeed irradiation and APS cause damage to endothelial cells, there is no reason why the extent or nature (reversible-irreversible) of this injury should be the same; ii. if the postulated injuries eventually resolve in cellular death, it is possible that the time it takes for the necrotic changes to develop and to be manifested is longer than the thrombocytopenic effect of single injections of APS, whereas by the time severe thrombocytopenia levels develop in the irradiation model, those necrotic changes have already developed and are ummasked in the absence of platelets; iii. the normally used amounts of APS serum do not arrest thrombopoietic activity, although they may affect magakaryocytes[26]; young larger platelets can be found in the circulation shortly after treatment with APS[28,46]. There is a possibility that those platelets may be, even in numbers too small to significantly raise platelet levels, more efficient in supporting endothelium than equal numbers of old, dwindling platelets in the thrombopoeitic arrest irradiation model of thrombocytopenia. The same difficulty of comparisons arises between fully irradiated and ineffectively irradiated animals, as it appears that perhaps even marginal

thrombopoeitic bone marrow activity can generate enough new platelets to alleviate the purpuric effect of thrombocytopenia.[22,23]

3. An Interpretation of Our Results

The lowering of the erythrocyte content in lymph past a first maximum can be due to either of the following, or a combination: 1. new platelets as discussed above coming into the circulation and reversing endothelial cell impairment; ii. the maximum is due to the combination of thrombocytopenia and the transient effect of vasoactive substances on the endothelium. These vasoactive substances (such as histamine and serotonin) are known to be released by platelets affected by APS[92,93] and have[89,90,91] a transient effect on vascular wall permeability inducing contraction of endothelial cells and the appearance of intercellular gaps through which cellular and subcellular plasma components could be extravasated. The dissipation of the effect of the vasoactive amines leaves a more stable baseline of vascular changes, due to the undernourishing and impairment of function of endothelial cells. It is this level of endothelial impairment that could further deteriorate if thrombocytopenia is sustained via repeated injections of APS.[54]

If indeed this second hypothesis is true and if our anti-endothelial cell activity free serum was truly free, then the lack of difference in the effect of the two sera we utilized (T and E groups) could be explained if we assume that, the controlling capillary-venules wall permeability factor in the first stage of APS induced thrombocytopenia was not endothelial thinning in the absence of platelets, but the effect of the vasoactive amines released by platelets affected by either sera. Since biologically equal APS doses were used and equal levels of thrombocytopenia were achieved, one could expect equal release reactions and equal capillaries-venules permeability changes, leading to comparable levels of extravasation of erythrocytes for the two sera in this first stage of APS thrombocytopenia.

4. Summary

We have unequivocally and quantitatively demonstrated, as manifested by increased RBC content in thoracic duct lymph, an increased extravasation of erythrocytes from the vascular compartment in anti-platelet serum induced thrombocytopenia. This experimental thrombocytopenic model appears to be a reversible one, as platelets can be transfused into the animals

and survive, shortly after the APS induction of severe thrombocytopenia. Thus, it appears that this model could be useful in certain aspects of platelet and pharmaceuticals research.

Also, in this model, it is rather unlikely that direct bleeding into the degenerating tissues of the lymph nodes, as may be the case in irradiation thrombocytopenic experiments, could result in artificially elevated estimates of the degree of the spontaneous extravasation of erythrocytes in thrombocytopenia. Furthermore, the fact that the animals are first operated on and then rendered thrombocytopenis, and the fact that the infusion of labelled RBCs takes place well into the post-operative period, minimizes the risk of direct bleeding into the abdominal cavity resulting in false high RBC concentrations in lymph measurements.

REFERENCES

1. Lacassagne, A. and Laveran, J. Numération des éléments du sang dans le syndrome purpurique du lapin nouveau-né. Compt. Rend. Soc. de Biol. 86: 713 (1922)

2. Shouse, S. S., Warren, S. L. and Whipple, G. H.: II. Aplasia of marrow and fatal intoxication in dogs produced by roentgen radiation of all bones. J. Exp. Med. 53: 421 (1931)

3. Liebow, A. A., Warren, S. and De Coursey, E. Pathology of atomic bomb casualties. Am. J. Path. 25: 853 (1949)

4. LeRoy, G. V. Hematology of atomic bomb casualties. Arch. Int. Med. 86: 691 (1950)

5. Cronkite, E. P., Jacobs, G.J., Brecher, G., and Dillard, G. The hemorrhagic syndrome of the acute radiation syndrome due to exposure of the whole body to penetrating ionizing radiation. Am. J. of Roentgenology, Radium Therapy and Nuclear Med. 67: 796 (1952)

6. Cronkite, E. P. Hemorrhagic syndrome of acute ionizing radiation illness produced in goats and swine by exposure to atomic bomb at Bikini, 1946. Blood. 5: 32 (1950)

7. Tullis, J. L. and Warren, S. Gross autopsy observations in animals exposed at Bikini. J.A.M.A. 134:

1155 (1947)

8. Kahn, J. B. Jr. and Furth, J. The pathogenesis of postisradiation anemia. Blood. 7: 404 (1952)
9. Wish, L., Furth, J., Sheppard, C. W. and Storey, R. H. Disappearance rate of tagged substances from the circulation of x-rayed animals. Am. J. Roentgenol. 67: 628 (1952)
10. Bigelow, R. R., Furth, J., Woods, M. C. and Storey, R. H. Endothelial damage by x-rays disclosed by lymph fistula studies. Proc. Soc. Exper. Biol. & Med. 76: 734 (1951)
11. Ross, M. H., Furth, J., Bigelow, R. R. Changes in cellular composition of the lymph caused by ionizing radiation. Blood. 7: 417 (1952)
12. Rosenthal, R. L. and Benedek. A. L. Blood coagulation and hemorrhage following total body x-irradiation in the rabbit. Am. J. of Physiology. 161: 505 (1950)
13. Cohn, S. H. Effects of total body x-irradiation on blood coagulation in the rat. Blood. 7: 225 (1952)
14. Cronkite, E. P. The hematology of ionizing radiation. In: Behrens, C. F. Atomic Medicine. New York, Thomas Nelson & Sons (1949)
15. Brecher, G. and Cronkite, E. P. Pathologic aspects of radiation hemorrhage and its prevention by platelet

transfusions. Proc. New York Path. Soc. 116, (1950-51)

16. Cronkite, E. P. and Brecker, G. Radio-activity: Effects of whole body irradiation. Ann. Rev. Med. 3: 193 (1952)
17. Jackson, D. P., Cronkite, E. P., Jacobs, G. J. and Behnens, C. F. Prothrombin utilization in radiation injury. Am. J. Physiol. 169: 208 (1952)
18. Woods, M. C., Gamble, F. N., Furth, J. and Bigelow, R. R. Control of postirradiation hemorrhagic state by platelet transfusion. Blood. 8: 545 (1953)
19. Spaet, T.H. Vascular factors in the pathogenesis of hemorrhagic syndromes. Blood. 7: 641 (1952)
20. Jackson, D. P., Sorensen, D. K., Cronkite, E. P., Bond, V. P. and Fliedner, T. M. Effectiveness of transfusions of fresh and lyophilized platelets in controlling bleeding due to thrombocytopenia. J. of Clinical Investigation. 38: 1689 (1959)
21. Roy, Albert J and Djerassi, Isaac. Proc. of the Society for Exp. Biol. and Med. 139: 137 (1972)
22. Aurnes, I. Appearance of red cells in peripheral lymphs during radiation-induced thrombocytopenia. Acta Physiol. Scand. 88: 392 (1973)
23. Aurnes, I. The vascular defect developing during thrombocytopenia. Bibliotheca Anatomica 12: 393 (1973)

24. Majno, G. Ultrastructure of the vascular membrane, in: Hamilton, W. F. (Ed): Handbook of Physiology, Sec. 2, Vol. III. Washington, American Physiological Society P. 2293 (1965)

25. Colman, W., Sievers, C. A., Pugh, R. P. Thrombocytopheresis: A rapid and effective approach to symptomatic thrombocytosis. J. Lab.Clin. Med. 68: 389 (1966)

26. Rolovic, Z., Baldini, M., Dameshek, W. Megakaryocytopoiesis in experimentally induced immune thrombocytopenia. Blood. 35: 173 (1970)

27. Evensen, S. A., Jeremic, M., Hjort, P.F. Experimental thrombocytopenia induced by busulphan (myleran) in rabbits: extremely low platelet levels and intact plasma clotting system. Thrombosis et Diathesis Haemorrhagica. 19: 570 (1968)

28. Mattison, W. E., Jr. Experimental thrombocytopenia and platelet agglutination. Bull. of the John Hopkins Hospital. 104: 63 (1959)

29. Marino.Recherches sur les plaquettes du sang. Compt. Rend. Soc. Biol. 58: 194 (1905)

30. Le Sourd, L. and Pagniez', P. L'irrétractilité du caillot et sa production expérimentale par action directe sur les hématoblastes. Compt. Rend. Soc. Biol. 61: 562 (1906)

31. Le Sourd, L. and Pagniez, P. Contribution a la question de l'origine des hematoblastes. Ibid. 63: 561 (1907)

32. Cole, R.J. Note on the production of an agglutimating serum for blood platelets. Bull. John Hopkins Hospital. 18: 261 (1907)

33. Chevrel and Roger. Isolement des hélmatoblastes - Production d'un serum antihematoblastique. Compt. Rend. Soc. Biol. 63: 501 (1907)

34. Sacerdotti, C. Archivio per le science mediche. 32: 339 (1908)

35. Stschastnyi. Blutplattchen und hämolytisches serum. Fol. Serol. 2: 285 (1909)

36. Aynaud, M. Globulins et serums antihematiques. Compt. Rend. Soc. Biol. 71: 697 (1911)

37. Ledingham, J. C. G. The experimental production of purpura in animals. Lancet 1: 1673 (1914)

38. Ledingham, J. C. G. and Bedson, S. P. Experimental purpura. Lancet 1: 311 (1915)

39. Lee, R. J. and Robertson, O. H. The effect of antiplatelet serum on blood platelets and the experimental production of purpura hemorrhagica. J. Med. Res. 33: 323 (1916)

40. Musser and Krumbhaar. The resistance of erythrocytes in normal rabbits and guinea-pigs and the changes produced in experimental purpura. J. Amer Med Assoc. 67: 1894 (1916)

41. Watabiki. Studies on experimental purpura hemorrhagica produced by anti-blood platelet serum. Kitasato Archives of Experimental Medicine. 1: 195 (1917)

42. Gottlieb. Experimental Purpura. J. Immunol. 4: 309 (1919)

43. Bedson, S. P. Blood platelet antiserum, its specificity and role in the experimental production of purpura, Part I. J. Path. Bacteriol. 24: 469 (1921)

44. Bedson, S. P. Blood-platelet anti-serum, its specificity and role in the experimental production of purpura, Part II. J. Path. Bact. 25: 94 (1922)

45. Tocantins, L. M. Experimental thrombopenic purpura in the dog. Arch. Path. 21: 68 (1936)

46. Tocantins, L. M. Experimental thrombopenic purpura; cytological and physical changes in the blood. Ann. Int. Med. 9: 838 (1936)

47. Vulpis. N. Experimental study on thrombocytopenic purpura induced by immune anti-platelet serum. Acta Haemat. 14: 72 (1955)

48. Humphrey, J. H. and Jaques, R. The release of histamine and 5-hydroxytryptamine (serotonin) from platelet by antigen-antibody reactions (in vitro). J. Physiol. 128: 9 (1955)

49. Ebbe, S., Baldini, M. and Dameshek, W. The antigenic structure of blood platelets; Histocompatibility antigens in rabbit blood platelets. Blood. 19: 548 (1962)

50. Henson, P. M. Release of vasoactive amines from rabbit platelets induced by antiplatelet antibody in the presence and absence of complement. J. Immunology. 104: 924 (1970)

51. Osler, A. G., Siraganian, R. P. Immunologic mechanisms of platelet damage. Progr. Allergy. 16: 450 (1972)

52. McDonald, T. P. and Clift, R. Mechanism of thrombocytopenia induced in mice by anti-platelet serum. Haemostasis. 5: 38 (1976)

53. Gore, I., Takada, M., Austin, J. Ultrastructural basis of experimental thrombocytopenic purpura. Archives of Pathology. 90: 197 (1970)

54. Kitchens, C. S., Weiss, L. Ultrastructural changes of endothelium associated with thrombocytopenia. Blood. 46 11: 567 (1975)

55. Moore, S., Friedman, R. J., Singlal, D. P., Gauldie, J., Blajchman, M. A., Roberts, R. S. Inhibition of injury induced thromboatherosclerotic lesions by anti-platelet serum in rabbits. Thrombos. Haemostas. 35: 70 (1976)

56. Levin, J., Cluff, L. E. Platelets and the Shwartzman phenomenon. J. of Experimental Medicine. 121: 235 (1965)

57. Hematology. Edited by W. J. Williams. McGraw-Hill Publications. (1972)

58. Hirsh, J., Doery, J. C. G. Platelet function in health and disease. Progress in Hematology. VII. Edited by Brown, E. B. and Moore, C. V. (1971)

59. Palade, G. E. Blood capillaries of the heart and other organs. Circulation. 24: 368 (1961)

60. Simionescu , M., Simionescu , N., and Palade, G. E. Morphometric data on the endothelium of blood capillaries. Journal of Cell Biology. 60: 128 (1974)

61. Albert, M. D. and Bucher, N. L. R. Latent injury and repair in rat liver induced to regenerate at intervals after x-irradiation. Cancer Res. 20: 1514 (1960)

62. Reinhold, H. S. Radiations and the microcirculation. Front. Radiat. Ther. Oncol. 6: 44 (1972)

63. Lindgren, A. L. and Riley, E.F. Use of the wound response to demonstrate latent radiation damage and recovery in x-irradiated intermitotic cells of the rat lens epithelium. Radiat. Res. 54: 411 (1973)

64. De Gowin, R. L., Lewis, L. D., Hoak. J. C., Mueller, A. L., Gibson, D. P. Radiosensitivity of human endothelial cells in culture. J. Lab. Clin. Med. 84: 42 (1974)

65. Lewis. L. J., Hoak. J. C., Maca, R. D. et al. Replcation of human endothelial cells in culture. Science. 181: 453 (1973)

66. Katsura, H. Experimental study on purpura of guinea pigs. Purpura by the antilemangioendothelial cell serum. J. Osaka M. A. 22: 373 (1923)

67. Clark, W. G., and Jacobs, E. Experimental non-thrombocytopenic vascular purpura: A review of the Japanese literature with preliminary confirmatory report. Blood. 5: 320 (1950)

68. Cronkite, E. P., Bond, V. P., Robertson, J. S., Paglia, D. E. The survival, distribution and apparent interaction with capillary endothelium of transfused radiosulfate labelled platelets in the rat. J. Clin. Invest. 36: 881 (1957)

69. Cronkite, Z. P., Bond, V.P., Fliedner, T. M., Paglia, D. A., Adamick, E. R. Studies on the origin, production and destruction of platelets. In: Blood Platelets. Johnson, S. A., Monto, R. W., Rebuck, J. W., and Horn, R. C., (Eds.). Boston, Little Brown Co. p. 595-609 (1961)

70. Johnson, S. A., Balboa, R. S., Bertram, H. D., Monto, R. W., Sregesmund, K. A. Greenwalt, T. J. The mechanism of the endothelial supporting function of intact platelets. Exper. and Molecular Pathology. 3: 115 (1964)

71. Wojcik, J. D., VanHorn, D. L., Webber, A. J., Johnson, S. A. Mechanism whereby platelets support the endothelium. Transfusion. 9: 324 (1969)

72. Rebuck. J. W., Blood platelets. Editorial, Transfusion. 3: 1 (1963)

73. Hager, E. B., Burns, R. O., Carter, R. D. and Merrill, J. P. Lesions produced in homografted canine kidneys by prior immunization with donor platelets. Fed. Proc. 21: 37 (1962)

74. Morrison, F. S., Baldini, M. G. Antigenic relationship between blood platelets and vascular endothelium. Blood. 33: 46 (1969)

75. Becker, C. G., Nachman, R. L. Contractile protein of platelets and endothelial cells. J. of Clinical

Investigation. 48: 7A (1969)

76. Becker, C. G. and Nachman, R. L. Contractile proteins of endothelial cells, platelets and smooth muscle. Am. J. of Pathology. 71: 1 (1973)

77. Gauldie, J. Assistant Professor of Pathology, Dept. of Immunology, McMaster University Medical Centre. Private Communications.

78. Mustard, J. F. and Packham, M. A. The reaction of blood to injury. In: Inflammation, Immunity and Hypersensitivity. H. Z. Movat (Ed.). Harper and Row (Pub.). New York. p. 527-607 (1971)

79. Saba, S. R. and Mason, R. G. Effects of platelets and certain platelet components on growth of cultured human endothelial cells. Thrombosis Research. 7: 807 (1975)

80. Tranzer, J. P. and Baumgartner, H. R. Filling gaps in the vascular endothelium with blood platelets. Nature. 216: 1126 (1967)

81. Ohsaka, A., Suzuki, K., Ohashi, M. The spurting of erythrocytes through junctions of the vascular endothelium treated with snake venom. Microvascular Research. 10: 208 (1975)

82. Van Horn, D. L., Johnson, S. A. The mechanism of thrombocytopenic bleeding. The Amer. J. of Clinical Pathology. 46: 804 (1966)

83. Van Horn, D. L., Johnson, S. A. The escape of carbon from intact capillaries in experimental thrombocytopenia. J. of Laboratory and Clinical Medicine. 71: 301 (1968)

84. Quick. A. J., E stein, E. Thromboplastic activity in human blood. J. Applied Physiol. 4: 840 (1952)

85. Danielli, J. F. Capillary permeability and oedema in perfused frog. J. Physiol. 98: 109 (1940)

86. Brettschneider, L., Kolff, J., Smith, G. V., Martin, A. J., Taylor, P., Starzl, T. E. An evaluation of perfusion constituents in liver preservation. Surg. Forum. 19: 354 (1968)

87. Gimbrone, M. A., Aster, R. H., Cotran, R. S., Corkery, J., Jandl, J. H., Folkman, J. Preservation of vascular integrity in organs perfused in vitro with a platelet rich medium. Nature (London). 222: 33 (1969)

88. McKay, D. G., Moroz, C., DeVries, A., Csavossy, I., Cruse, V. The action of hemorrhagin and phosphalipase derived from vipera palestinae venom on the microcirculation. Lab. Investigation. 22: 387 (1970)

89. Majno, G., Shea, S. M., Leventhal, M. Endothelial contraction induced by histamine type mediators. The Journal of Cell Biology. 42: 647 (1969)

90. Majno, G., Ryan, G. B., Gabbiani, G., Hirschel, B. J., Irlé, C., Joris , I. Contractile events in inflammation and repair. In: Inflammation, Mechanisms and Control. LePow, J. H., Ward, P. A., (Eds.). Academic Press. p. 13 (1972)

91. Joyner, W. L., Carter, R. D., Raizes, G. S., Renkin, E. M. Influence of histamine and some other substances on blood-lymph transport of plasma protein and dextran in the dog paw. Microvascular Research. 7: 19 (1974)

92. Henson, P. M., Cochrane, C. G. In: Cellular and Humoral Mechanisms of Anaphylaxis and Allergy. Edited by H. Z. Movat: S. Karger, Basel, p. 29 (1969)

93. Henson, P. M. Release of vasoactive amines from rabbit platelets induced by antiplatelet antibody in the presence and absence of complement. Journal of

Immunology. 104: 924 (1970)

94. McDonald, T. P. and Clift, R. Mechanism of thrombocytopenia induced in mice by anti-platelet serum. Haemostasis. 5: 38 (1976)

95. Karpatkin, S. Human platelet senescence. Ann. Rev. Med. 23: 128 (1972)

96. Buonassisi, V. Sulfated mucopoly saccharide synthesis and secretion in endothelial cell cultures. Exp. Cell. Res. 76: 363 (1973)

97. Buonassisi, V. and Root, M. Enzymatic degradation of heparin-related mucopolysaccharides from the surface of endothelial cell cultures. Biochim. Biophys. Acta. 385: 1 (1975)

98. Jaffe, E. A., Minick, C. R., Adelma, B., Becker, C. G. and Nachman, R. Synthesis of basement membrane collagen by cultured human endothelial cells. Journal of Exper. Medicine. 144: 209 (1976)

99. Majno, G. and Palade, G. E. Studies on inflammation, I. The effect of histamine and serotonin on vascular permeability: an electron microscopic study. J. Biophys. Biochem. Cytol. 11: 607 (1961)

100. Majno, G. Mechanisms of abnormal vascular permeability in acute inflammation. In: Injury, inflammation and Immunity. Edited by Thomas, L., Uhr, J. W., Grant, L.: Baltimore: Williams and Wilkins. p. 58-93 (1964)

101. Movat, H. Z. and Fernando, N. V. P. Allergic inflammation, I. The earliest fine structural changes inthe blood tissue barrier during antigen-antibody interaction. Am. J. Pathology. 42: 41 (1963)

102. Moore, S., Friedman, R. J., Singal, D. P., Gauldie, J., Blajchman, M. A., Roberts, R. S. Inhibition of injury induced thromboatherosclerotic lesions by anti-platelet serum in rabbits. Thrombos. Haemost. (Stuttg). 35: 70 (1976)

103. Buchanan, M., Ph. D. Candidate., Dr. J. Hirsh, Professor of Pathology. Medical Sciences, McMaster University. Private consultations.

104. Ardlie, N. G., Packham, M. A. and Mustard, J. F. Adenosine diplosphate induced platelet aggregation in suspensions of washed rabbit platelets. Br. J. of Haem. 19:7 (1970)

105. Dillard, G. H. L., Brecher, G., Cronkite, E. P.

Separation, concentration and Transfusion of platelets. Proc. Soc. for Experiment. Biology and Medicine. 78: 796 (1951)

106. Brecher, G. and Cronkite, E. P. Morphology and enumeration of human blood platelets. J. of Appl. Physiol. 3: 365 (1950)

107. Brecher, G., Schneiderman, M., Cronkite, E. P. The reproducibility and constancy of the platelet count. Am. J. of Clin. Path. 23: 15 (1953)

108. Bollman, J. L., Cain, J. C., and Grindlay, J. H. Techniques for the collection of lymph from the liver, small intestine, or thoracic duct of the rat. J. Lab. Clin. Med. 33: 1349 (1948)

109. Bienenstock, J. Professor of Medicine, McMaster University School of Medicine. This technique, as used in the Immun. lab., was demonstrated to us by his technical assistants at his request. Also, private consultation on differential lysing of mixtures of lymplocytes and erythrocytes.

110. Coulter Counter Electronics, Inc, Hialeah, Florida, U. S. A. Equipment manual with extensive bibliography. Of particular interest are the following references.

111. Brecher, A., Schneiderman, M., Williams, G. Z. Evaluation of electronic red blood cell counter. J. of Clinical Pathology. 26: 1439, December (1956)

112. Magath, T. B., Berkson, J. Electronic blood cell counting. Am. J. of Clin. Path. 34: 203 Sept. (1960)

113. VanderBoom, John, Chief Technologist, Routine Hematology Laboratory, McMaster University Medical Centre.

114. Mittel, K. K., Mickey, M. R., Singal, D. P., and Terasaki, P. I. Serotyping for homotransplantation. XVIII. Refinement of microdroplet lymphocyte cytotoxicity test. Transplantation. 6: 913 (1968)

115. Webber, C. E. and Associates. Assistant Professor of Radiology, McMaster University Medical Centre.

116. Gray, S. J., Sterling, K. The tagging of red cells and plasma proteins with radioactive chromium. J. of Clin. Invest. 29: 1604 (1950)

117. Donohue, D. M., Motulsky, A. G., Giblett, E. R., Pirzio-Biroli, G., Viranuvatti, V., Finch, C. A. The use of chromium as a red-cell tag. British Journal of Haematology. 1: 249 (1955)

118. Mollison, P. L. Blood Transfusion in Clinical Medicine. Fifth Ed. Blackwell Scientific Publications. P. 100-102, 104, 683-685 (1972)

119. The International Committee for Standardization in Hematology: Recommended Methods for Radioisotope red cell survival Studies. Nuclear Medicine 11: 352-361 (1972)

120. Ebaugh, F. G., Emerson, C. P., and Ross, J. F. The use of radioactive chromium 51 as an erythrocyte tagging agent for the determination of red cell survival in vivo. J. Clin. Invest. 32: 1260 (1953)

121. Clarke, G. M. Statistics and Experimental Design. (Contemporary Biology Series). Edward Arnold Ltd., London, England (1969)

122. Bach, C. and Lewis, G. P. Lymph flow and lymph protein concentration in the skin and muscle of the rabbit hind limb. J. Physiol. 235: 477 (1973)