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Some Important Milestones in the Field of Blood Clotting

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Abstract
Several different kinds of 'milestone' in the field of blood coagulation are described from the middle decades of the 20th century. Although viewed from the standpoint of clotting per se, attention is also given to implications for innate immunity. The first milestone considered is the protracted saga of clotting dependence on vitamin K, an adventure that spanned more than five decades beginning in the 1920s. The second has to do with the discovery of a half-dozen 'new' clotting factors during the period immediately following World War II. A third pursues a narrower focus and examines the once mysterious transformation of fibrinogen into fibrin. Finally, the clinical treatment of classical hemophilia had a remarkable turning point in the 1960s as the result of simple but sensible measures.

Introduction

In their invitation to summarize some important milestones in the field of blood clotting, given its relationship to the immune system, the editors mentioned a time frame of 'over the last century'. To that end I have chosen several quite different advances, all of which occurred in the middle decades of the 20th century before cloning and recombinant technology. In the way of a caveat, it must be agreed that what may be regarded as a 'milestone' by the blood clotting community may only rank as a common wooden post by researchers of innate immunity. Nevertheless, I have tried to show how most of these advances set the stage for appreciating the common ground of injury, infection and inflammation.

We can also agree that the path to any milestone encompasses a great many steps trudged by numerous researchers, even if only a few are mentioned in the end. Readers interested in a broader rendering of significant twentieth century events in the field of blood clotting may want to consult the excellent compendium initially undertaken by Charles Owen and ultimately and consummately edited by Nichols and Bowie [1], or, for a more personal and very readable chronicle of discovery, the small book by Hougie [2].

The Vitamin K Story

The tale begins with two independent and not quite serendipitous findings in the 1920s. Two different groups of animals were found to be hemorrhagic, one as a result of something they were eating and the other as a result of something they were *not* eating. The two stories, although often told separately and narrowly, are remarkably intertwined and parallel. In both cases searches were quickly

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begun to isolate the active principal and determine its chemical nature. In fact, each was needed for finding what the other was doing.

The first began when it was recognized by farmers in certain parts of North America that the 'sweet clover disease' causing their cattle to bleed to death was *spoiled* sweet clover. Indeed, the disease could be reversed by feeding fresh sweet clover instead of the spoiled stuff. Sweet clover gets its name from the pleasant odor of newly mown hay, already attributed to a chemical called coumarin. At the time, blood clotting was thought to involve only four components: fibrinogen, prothrombin, a tissue-based prothrombinase dubbed thromboplastin, and calcium ions. As early as 1931 it was reported that the afflicted cattle had a prothrombin deficit [3].

Meanwhile, in the late 1920s Henrik Dam was feeding a cholesterol-free synthetic diet to chicks as part of a study on the synthesis of cholesterol [4], and he soon recognized that the chicks were becoming hemorrhagic. Adding cholesterol to the synthetic diet did not help, and Dam realized that something else must be missing from the bland mixture of starch, yeast extract and salts that he was using. Thinking that the symptoms were similar to scurvy [5], he added in vitamin C but found no benefit. Convinced the missing ingredient was vitamin-like, he tried all known vitamins, water-soluble and fat-soluble alike, to no avail. What did work were ether-extracts of leafy vegetables, leading him to proclaim the substance was a fat-soluble vitamin. He named it vitamin K, in line with the Scandinavian spelling of Koagulation [6]. Subsequently, vitamin K was crystallized and characterized by Edmond Doisy and his colleagues in 1938 [7].

Back on the other track, Karl Link and his coworkers in Wisconsin were trying to find the substance in spoiled sweet clover that was causing cattle to become hemorrhagic. After several years of effort, it turned out to be dicoumarol, a chemical that resembled the recently characterized vitamin K. The compound was apparently being produced by a fungus oxidizing coumarin in the sweet clover. Although dicoumarol was clearly an antagonist of vitamin K, plain coumarin was not (fig. 1). The Link group crystallized and characterized the poisonous substance just about a year after the report by Doisy on vitamin K [8].

Although Dam and Doisy were awarded the 1943 Nobel Prize in Biology and Medicine for their work on vitamin K, arguably it was the discovery and characterization of the inhibitor that was to have the greater impact on mankind. Unlike the vitamin, which was known to exist in two similar but equivalent forms, the naturally occurring anticoagulant could be improved upon, and an intense search for more potent synthetic inhibitors was quickly undertaken [9]. The most remarkable of these turned out to be warfarin (fig. 1), named for the Wisconsin Alumni Research Fund that paid for the work; it was a compound destined to be administered to millions of people over the next half century (often under the commercial name of Coumadin). Moreover, warfarin was also a first-order rodenticide (rat poison) that would be manufactured by the megaton.

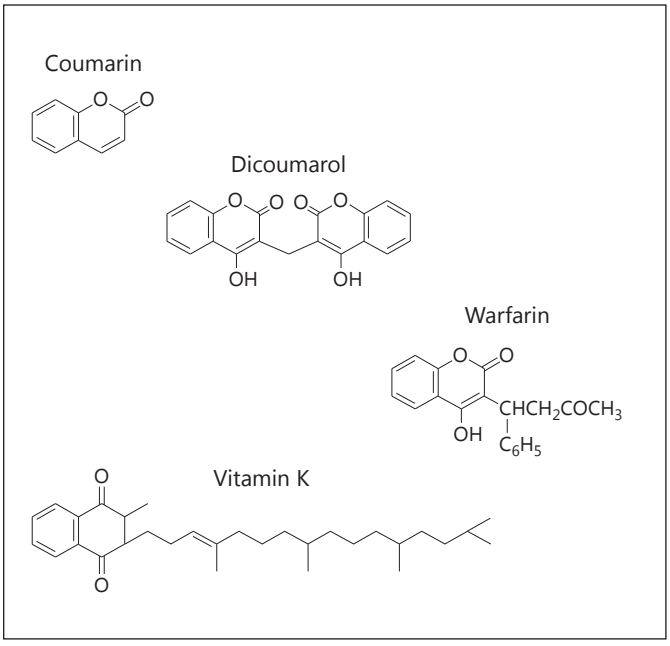

Fig. 1. Structures of coumarin and related substances.

As we shall see, warfarin was also vitally important in the discovery of a series of new blood clotting factors related to prothrombin, including factors VII, IX and X (and later protein C). What remained elusive for a very long time, however, was just how vitamin K was exerting its influence and how dicoumarol and its relatives were preventing blood clotting. Early experiments ruled out the vitamin being an integral part of prothrombin or the other factors. Instead, it seemed the dicoumarol-like inhibitors were in some way interfering with the synthesis of those proteins.

It was not until 1968 that it was found that the blood plasma of persons being treated with warfarin contained an immunochemically recognizable protein very much like prothrombin, even though there was no demonstrable prothrombin clotting activity [10]. Not long after, Johan Stenflo, working on his PhD dissertation in Lund,

Sweden, switched to dicoumarol-treated cows in order to have more blood for purification purposes [11], a move quickly followed by workers in North America [12]. The large amount of plasma needed for purification purposes was a necessity in those days before recombinant DNA technology. Stenflo found that dicoumarol administration led to a situation in cows similar to that which had been found in humans, an inactive prothrombin that was immunochemically similar to prothrombin but biologically inactive. Moreover, the protein was electrophoretically distinguishable from normal prothrombin [11]. Very soon thereafter, in another insightful experiment, Shah and Suttie [13] injected vitamin K-deficient rats with radioactive amino acids and showed that the action of vitamin K was to convert an inactive precursor version of prothrombin to an active one.

The climax came when key peptides from authentic prothrombin were compared with the corresponding peptides from the 'prothrombin' of dicoumarol-treated cows. In fact, a set of peptides from the active protein was much more negative than the comparable peptides from the treated cows. Mass spectrometry showed the presence of γ-carboxyglutamic acid, now commonly referred to as 'GLA'. These posttranslationally formed amino acids are decomposed into ordinary glutamic acid under conditions of routine acid hydrolysis and had been missed during several decades of study on prothrombin. The first GLA report was received from Stenflo's laboratory in May, 1974 [14] and independently submitted only a month later by workers in Minnesota [15], where sweet clover disease had first been observed and studied.

The existence of GLA residues revealed the basis of the tight binding of calcium by prothrombin and other vitamin K-dependent factors that was later found to be needed for assembling these factors on the surfaces of activated blood platelets. It also explained why these proteins are selectively adsorbed by insoluble salts like barium sulfate. Subsequent studies showed that there is a rather complex biosynthetic cycle in play, with the vitamin K acting as a cofactor for the gamma carboxylase that forms the GLA residues actually existing in the dihydroquinone (reduced) form before being oxidized to an epoxide during the carboxylation. The epoxide needs to be changed back into simple vitamin K for the cycle to function, and it is this enzyme, the vitamin K epoxide reductase, that is inhibited by dicoumarol. Remarkably, different mutations in this enzyme can lead to a combined deficiency of all vitamin K-dependent factors on the one hand, or warfarin resistance, by different mutations, on the other [16].

More and More Clotting Factors and Confusion

Not all the 20th century milestones occurred exclusively in laboratories. One was largely due to, of all things, an international nomenclature committee, the deliberations and judgments of which went a long way towards providing an understanding, or at least a pathway to an understanding, of the complex nature of blood clotting. It was what might be called a 'time of realization'.

As noted above, before World War II the common view of blood clotting involved a four-component system, even if a few researchers were already aware there had to more to it. The four necessary components – fibrinogen, prothrombin, tissue thromboplastin and calcium ions – could be reassembled in vitro in a simple assay to test the quality of a person's blood clotting. Commercial thromboplastin prepared from rabbit brain tissue was available and, when combined with a measured amount of a subject's blood plasma (the source of prothrombin and fibrinogen) and a specified quantity of calcium as a trigger, gave quite reproducible clotting times. Because the amount of prothrombin was thought to be limiting, these were called prothrombin times. Typically, persons with a tendency to bleed had long prothrombin times.

It was also known by then that prothrombin could be selectively removed from plasma simply by adsorbing it on to any of several group II insoluble salts like barium sulfate, and its activity could be recovered by elution from the barium sulfate with citrate. It was also known that calcium ion, a group II element, bound to and was intimately involved with prothrombin.

It was late in World War II when a fifth component, discovered by Paul Owren [17], ignited what is now known as a 'paradigm shift'. A woman with severe bleeding was admitted to a hospital in Norway, and, when a standard prothrombin assay was conducted, her plasma was found to have a greatly delayed 'prothrombin time'. She was given a prothrombin concentrate but it did not help, nor did fibrinogen. However, a tiny amount of normal plasma corrected her 'prothrombin time'. Moreover, her own plasma could substitute in tests of other plasmas that used barium sulfate-adsorbed normal plasma. Because the thromboplastin component in these tests came from normal rabbit brains, and because calcium is calcium, Owren concluded she must be lacking some other component, a fifth factor he dubbed factor V. It was the first of several new factors that would be discovered in 1940s and 1950s.

As these events were unfolding in Norway, a prominent clotting specialist in North America, Armand Quick,

Table 1. Researchers most credited with respective discovery milestones

Milestone	Principal researchers	Year(s)
Discoveries of vitamin K and dicoumarol	H. Dam, K. Link, E. Doisy	1928–1943
Discovery of γ-carboxyglutamic acid	P.O. Ganrot, J. Stenflo, J. Suttie, G. Nelsestuen	1968–1974
Discovery of numerous clotting factors and the need for systematic nomenclature	P. Owren, C. Owen, B. Alexander, R. Biggs, C. Hougie, F.-H. Duckert, F. Koller and others; International Committee on Nomenclature	1943–1960
The molecular basis of fibrin formation	K. Laki, E. Mihalyi, K. Bailey, L. Lorand, J. Ferry	1950–1952
Discovery of cryoglobulin as an administrable source of factor VIII	J. Pool	1959–1964

the originator of the 'prothrombin time' test, had found what he felt were two forms of prothrombin, which he called A and B [18]. When the war was over and journal publications caught up with events, Owren was able to show that Quick's prothrombin A was actually factor V [19]. Shortly thereafter, Owren reported that he had found yet another factor, which he called factor VI [20].

Meanwhile, other new cases of individuals with unexplained bleeding were being reported, for most of which small amounts of normal plasma were able to remedy clotting delays in vitro. In one case, a small amount of normal serum (as opposed to plasma) was found to be sufficient [21]. Ordinarily, when blood or plasma is clotted in vitro all the prothrombin is consumed, but now it was found that a factor had survived the clotting process. This 'stable factor' came to be called to be called factor VII [22], in line with Owren's use of Roman numerals; it was later found to be directly activated by a tissue factor. Like prothrombin, it was adsorbed onto the salts of the diatomaceous earths (e.g. barium sulfate). In fact, the newly discovered factors were falling into two classes: those that were adsorbed onto salts like barium sulfate and resembled prothrombin, and those like factor V that did not. The ones that were adsorbed on to barium sulfate, which by 1960 included at least three more factors, were presumed to be related to prothrombin and similarly involved with calcium ions.

As the new factors were becoming more legitimately recognized, it became apparent that there were two quite different pathways leading to thrombin generation, one of which seemed not dependent on tissue damage and was managed with components that were 'intrinsic' to the blood. So far as can be determined, the first declaration of 'intrinsic' and 'extrinsic' pathways leading to a common activation of prothrombin was made by D.E. Bergsagel in his doctoral thesis at Oxford in 1955 and cited as a 'personal communication' in a 1957 article [23]. During this period, as we shall see, connections between blood clotting and inflammation were also becoming more obvious.

There was initially much confusion, with the new clotting factors being named in a helter-skelter fashion. Lest the reader think this is an exaggeration, here is an exact quote from a 1960 report describing the situation [24]: 'Two of the (new) blood clotting factors were described by 13 different terms, and a third by 12; one factor had been given four different names by a single investigator.'

It was the chaotic and inconsistent naming of new factors that led to the formation of the International Committee on Nomenclature of Blood Clotting Factors and their systematic designation by Roman numerals. In 1959 the committee issued a declaration that was published simultaneously in numerous journals around the world [25]. In these announcements the committee formalized the use of Roman numerals, making factor VII the official designation for the stable serum factor, and establishing factors VIII and IX for two factors associated with hemophilia. Additionally, it formally proclaimed that factor VIII, previously called antihemophilic globulin or antihemophilic factor, is responsible for classical hemophilia, now to be called hemophilia A, whereas hemophilia B, also a sex-linked disease, is caused by a deficiency of factor IX.

Not all the confusion was immediately dispelled, especially when it was found that factor VI was actually an activated form of factor V. In addition to the confusion about naming, the priority of who exactly discovered which factor was also to a degree muddled. Some of the researchers most credited with at least one of the new factors are named in table 1. More reliable details can be found in Owen [1].

Not all the claimed factors made the Committee's 1959 list, even though good evidence was already in hand for some of them. These included a vitamin K-dependent factor that was later to be designated factor X, the important common agent serving as the junction point for the extrinsic and intrinsic pathways. Similarly, the 'contact' factors XI and XII – both serine proteases but not vitamin K-dependent – were also left off the original list, being added along with factor X in a 1962 update [26].

Factor XI had previously been called plasma thromboplastin antecedent or PTA [27], and factor XII was until then known as Hageman factor [28]; the two were postulated to be the initiators of the intrinsic system and the activator of factor IX, which *is* vitamin K-dependent (fig. 2). One of the reasons the Committee had hesitated about their importance to clotting was that the absence of Hageman factor (factor XII), although exhibiting a prolonged whole blood clotting time in vitro, did not cause unusual bleeding [29]. Indeed, the prothrombin time, an in vitro test, was also normal. In contrast, factor XI deficiency does cause bleeding, even though it was clearly being activated by factor XII. It later became apparent that factor XI can also be activated by thrombin. It is also noteworthy that the close evolutionary relative of factor XI called prekallikrein, the activated form of which releases bradykinin from high molecular weight kininogen (HK), is *not* activated by thrombin. Thus, factor XII and HK both bind to negatively charged surfaces, and factor XI and prekallikrein associate with HK. As such, the contact factor system can provoke both clotting and inflammation, the latter in the form of bradykinin release (fig. 2). It is now known that the 'negatively charged substances' can include the lipopolysaccharides of Gram-negative bacteria.

By late 1950s it was already widely known that mammalian blood plasma develops the ability to induce pain and increase vascular permeability following its exposure to glass surfaces [30]. Remarkably, it was found that the plasma of persons lacking factor XII (still Hageman factor at the time) did not exhibit those phenomena [31]. The pain and permeability responses were mainly due to the release of kinins, themselves hallmarks of inflammation (fig. 2).

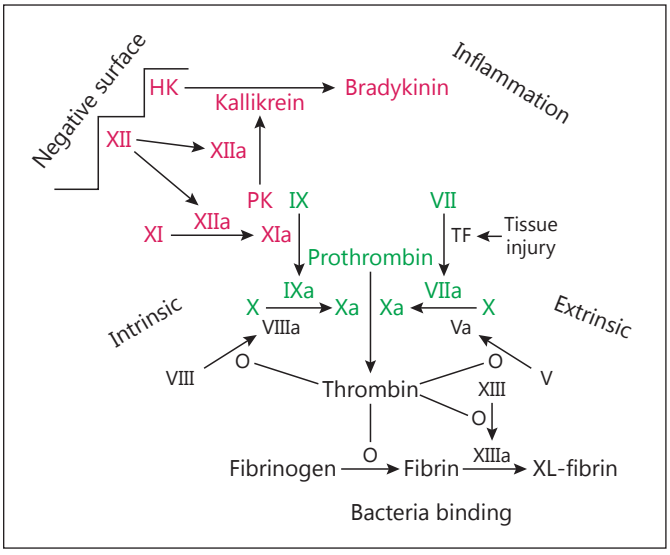

Fig. 2. Pathway convergences for clotting and inflammation. Contact factors are red; vitamin K-dependent factors are green. PK = Prekallikrein; XL = cross-linked.

The Conversion of Fibrinogen to Fibrin

A milestone of a quite different sort was reached during a brief period not long after World War II. In this case, the efforts of several laboratories provided complementary data that gave rise to a remarkably prescient model of how the polymeric gel we call fibrin actually forms. Much of the magic of how an enzyme can transform a soluble protein like fibrinogen into a rubbery gel was revealed.

Although Alexander Schmidt had shown as long ago as 1872 that thrombin was an enzyme that converted fibrinogen to fibrin, and although over the years several authors suggested, not convincingly, that proteolysis might be involved [described in 32], it is the more recent history that we need to focus on. Thus, in 1937 Eagle and Harris [33] showed that papain, a well-characterized protease, could gel fibrinogen similar to what happened with thrombin. The question remained, how can cutting a protein into pieces transform it into a polymeric gel?

In the end, a series of independent and complementary observations from several laboratories explained the process very well. In the first of these, Koloman Laki [34] showed that fibrinogen treated with thrombin at a low pH does not clot, but upon neutralization the system instantly gels. Even before that, Elemer Mihalyi [35] had convincingly demonstrated that fibrinogen and fibrin dispersed in urea have different electrophoretic mobilities, with fibrinogen being more electronegative than fibrin. Finally, the laboratories of Kenneth Bailey and Lazlo Lorand independently performed crucial end-group studies that showed the differences between fibrinogen and fibrin, publishing a joint paper in 1951 [36]. It was all summed up elegantly by John Ferry [37] in a classic 1952 article. The simple notation belied the accompanying ex-

planation of how limited proteolysis and the removal of some negatively charged patches on fibrinogen led to an intermediate that spontaneously polymerized in an overlapping fashion to give a two-molecule-thick protofibril.

The proposal held up to a half-century's scrutiny by an army of researchers, who poked, prodded and tested fibrinogen and fibrin from every possible vantage point, beginning with electron microscopy and ending with high-resolution X-ray crystallography. During this period, the final Roman numeral was also assigned, factor XIII being shown to be a transglutaminase that introduced cross-links between the units in fibrin. Additionally, more and more experiments were reported showing how pathogenic bacteria, including particular strains of staphylococci [38] and streptococci [39], bound to fibrinogen or fibrin, with the advantage to the host or pathogen not always being clear. Also, even though fibrin is fundamentally only a polymer of a slightly modified fibrinogen, packing and subtle structural adjustments lead to a host of unique properties, including it being a chemoattractant for a variety of white cells that participate in inflammation.

Of course, fibrinogen is an abundant protein in plasma and is itself very amenable to the physicochemical characterization that led to this detailed understanding. It is also one of the least soluble proteins in plasma and as a result is easily prepared by various simple precipitation schemes. Many of the other clotting factors are present in only minute quantities, however, and the prospect of purifying some of them for such mechanistic studies seemed hopeless. Probably the most sought after and greatest source of frustration at the time was factor VIII, mutations in the gene of which lead to classical hemophilia.

Cryoglobulin and the Treatment of Hemophilia

Classical hemophilia was the longtime scourge of the blood clotting field, the sex-linked genetics of which were appreciated centuries ago. By the middle of the 20th century this crippling disease led to so many hospitalizations in developed countries that many families simply relocated to be near major blood centers in cities like Oxford, Stockholm, Boston or Chapel Hill, North Carolina. The afflicted children, virtually all boys, were most often crippled and on crutches, the result of internal bleeding at joints like the knees or hips. For a long time the standard treatment had been a whole blood transfusion. The protein responsible for the problem, factor VIII, is present in the blood plasma in minute quantities and was resisting determined efforts at purification.

By the mid-1950s it was known that the active principal in plasma tended to precipitate with fibrinogen by various procedures, and such fractions were a frequent starting point for purification efforts [40]. For convenience, preparations were begun with plasma that had been frozen rather than fresh material. It was common knowledge that fibrinogen was slow to go back into solution during the thawing of the plasma, and it seemed reasonable to remove that still insoluble material by centrifugation as a second simple step for getting a head start on obtaining factor VIII. It was Judith Pool who first tried this and was disappointed to find that the supernatant plasma had no factor VIII activity at all [41]. However, she was thrilled to find that one hundred percent of the factor VIII activity was in the sedimented fraction that was slow to dissolve after thawing. Chance favors the prepared mind, and Pool saw here an opportunity to convert a handicap into an advantage. With great determination, she turned her efforts to preparing a form of the cryoprecipitate that could be administered directly to patients with classical hemophilia. It was a revolution.

First, Pool found that the activity in the cryoprecipitate could be redissolved in small amounts of sterile saline and safely injected [42]. Furthermore, the antihemophilic activity was greatly concentrated in the process and it could be safely frozen and stored in a home freezer without loss of activity. She and her colleagues worked out every detail, showing how plasma could be collected at a blood bank in a simple collection bag, centrifuged, the red cells diverted, the plasma frozen and then thawed, another centrifugation, the plasma diverted, and the cryoglobulin then stored, still in the original collection bag [43]. The redissolved material was 30-fold more potent on a volume basis than fresh plasma. As we shall see, this system of a single-unit process had one other very important advantage over methods that began with pooled plasma.

Administration of this simple preparation, simply called 'cryo' for cryoprecipitate, furnished immediate relief and protection against hemorrhage for most patients with classical hemophilia. Moreover, because it could be packaged and stored at home, patients were taught to self-administer. The material was soon being mass-produced and within only a few years the crutches had disappeared. It was a genuine milestone.

Unhappily, Pool died in 1974 at the age of 56. If she had lived longer, she might have resisted the commercial drive that subsequently abandoned her single-bag approach in favor of mass production and the use of vast pools of plasma. It was these that gave rise to tragedies in the way of widespread hepatitis and HIV infection among hemophiliacs, unintended consequences that would later

be overcome by the introduction of recombinant DNA technology and its own era of milestones that can be chronicled on another occasion.

In summary, I have described some highlights in the field of blood clotting that occurred in a period half a century ago. Some of the developments were provoked by environmental factors, others by an appreciation of genetic differences among persons with bleeding tendencies. One was driven by simple curiosity to understand gel formation, and another was inspired by a chance to relieve suffering among those afflicted by classical hemophilia. There were doubtless other milestones among the many advances that occurred during that age of realization, but these were a set that ought to rank high on any list.

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