The HLB Blood Test as an Indicator of Oxidative Injury & Disseminated Intravascular Coagulation

by Robert W. Bradford, DSc and Henry W. Allen
Bradford Research Institute, Chula Vista, California

Introduction and Historical Perspective

More than 150 years ago a British physician, T.W. Jones, asked the question, "Why does the blood circulating in the vessels not coagulate?"[1] Or, stated in reverse, Why does the blood coagulate once it leaves the vessels? Though the biochemical mechanisms involved in answering each of these questions differ, the final answers have not as yet been found after 150 years of diligent research.

Almost 130 years ago[2] a French physician, A. Trousseau, recognized that the blood of cancer patients is in a hypercoaguble state and is in the process of clotting even while coursing through the veins. The name that has been given to this discovery is still in use today as "Trousseau's Syndrome."

Early in his career, Rudolph Virchow, the Father of Pathology, was interested in thrombosis and embolism.[3] Virchow speculated that blood could be altered in the body so that it would clot as a result of a stimulus too weak to clot normal blood. In 1845 Virchow delivered a lecture setting forth this concept.

Thus, it may easily be seen that the not-so-obvious concept that blood may partially clot in living persons reaches back into the very beginning of modern medicine.

The discovery of the biochemical mechanisms of blood clotting has been different from the development of many other areas of science, much of it being left to chance. Basically, the blood clotting process may be simply described as the formation of chains of units (links) which are then cross-connected to form a 3-dimensional network or hard clot. The link forming these chains is itself always present in the blood but in an inactive form and must be activated before chain formation can occur. It is the activation of this unit (fibrinogen) which is of such great complexity. Blood may be stimulated to clot by many different pathways and for many different reasons. Regardless of the initiating factors, the blood clotting process is represented as a sequence of events in which the activation of one factor triggers the activation of another until, after a series of separate steps, the final goal of fibrinogen activation occurs.

The discovery of the various blood clotting factors or proteins which, when activated, lead to fibrinogen activation, were for the most part discovered by chance. The admission of a patient into the hospital with an unexplainable bleeding disorder attracted blood researchers to try to discover the cause of the hemorrhaging. Analysis of blood from normal persons led to the discovery of several of these factors, working backwards from fibrinogen (Factor I). When a patient with an unusual bleeding disorder was studied, a new previously unrecognized factor was discovered which was missing from that person's blood. For this reason several of the blood clotting factors have been named after the individuals in which they were found missing. For example, Christmas factor (Factor IX), Hageman factor (Factor XII), etc.

There will be described the causes of partial blood clotting in pathological states as well as the detection of this condition by the HLB Blood Test and medical miroscopy. Also described are the possibilities for treating this difficult-to-diagnose disease.

The Mechanics of Blood Clotting

Blood clotting is a mechanistic process, highly detailed and involving many separate and distinct components. The problem for hematologists has been to understand the mechanisms involved at the biochemical level. Undoubtedly, efforts to fully understand blood clotting mechanisms will continue for many more years.

There will be described only those aspects of blood clotting as they pertain to the abnormal clotting of blood in degenerative diseases and infection (sepsis), the detection of abnormal blood clotting as revealed by the HLB Blood Test and the treatment of these pathologies.

As previously mentioned, the clotting of blood involves linking end to end (polymerization) a fundamental protein unit with the formation of a chain of these units (fibrin). The completion of this first step, the formation of fibrin, leads to a product which is still soluble. Crosslinking these strands into a network (by Factor XIII) results in what is called the "hard clot."

Fibrinogen (the basic unit) which has linked together into fibrin (the chain) may or may not be further processed (crosslinked) to form an insoluble precipitate (hard clot). Those who are or are capable of forming fibrin from fibrinogen are said to be in a "hypercoaguable" state while those, for one reason or another, who have a diminished ability to form clots are in a "hypocoaguble" state.[4]

When blood is clotted, the factors that are involved are consumed, that is, incorporated into the clotted blood itself. The body then recognizes that there is a deficiency of clotting agents and generates more. Thus, one who has blood that tends to clot excessively will alternate between a hypercoaguable state and a hypocoaguable state. When in the hypocoaguable state such people hemorrhage until the deficient clotting factors are replaced.[5]

When only soluble fibrin is formed from fibrinogen there may be experienced some difficulty in detection. It may be detected by a change in blood viscosity (sedimentation rate), by the HLB Blood Test (described more fully below) or by other more subtle means. This form of hypercoagulation may not be visible in other ways and may go undetected.

If, however, soluble strands of fibrin are crosslinked, an insoluble precipitate of sometimes microscopic fibers results, which may be detected in the microscope. An excessive formation of insoluble, crosslinked fibrin leads to an impairment in circulation and eventual organ failure.[6]

With this background we are in a position to understand the name that has been given to the hypercoaguable

state, disseminated intravascular coagulation or DIC. This term refers to the pathology of the ability of the blood to coagulate, thereby forming soluble and/or insoluble polymerized fibrinogen.

The Significance of Oxygen Metabolism in DIC

Intermeshed with many of the mechanisms for DIC in various pathological conditions are the by-products of oxygen metabolism. What follows is a shortened description of the definition, origin and significance of several of the Reactive Oxygen Toxic Species, or ROTS, involved in the causative reactions leading to DIC. These reactive and toxic metabolites of oxygen are known by various names and will be referred to as "oxidative stress," an "oxidative burst" (of leukocytes), "free radical pathology," or simply ROTS.

The utilization of oxygen by the body leads to the formation of several by-products which may, if not immediately destroyed by specific enzymes, react among themselves to produce additional forms which are more toxic than the original substances.

One such substance is superoxide which is a form of oxygen that has not only bound an electron but become a highly damaging substance known as a "free radical." Free radicals result whenever molecules are broken apart and the fragments will make every effort to reunite with other molecules. When reunited, the molecule reacted with is damaged and is no longer the same substance. Because of the free radical and highly reactive aspect of superoxide, nature has provided a means of eliminating this by-product of oxygen metabolism almost as soon as formed. The control for superoxide is the enzyme superoxide dismutase (SOD) found in red blood cells and almost all cells utilizing oxygen.

A second member of the ROTS family is hydrogen peroxide, which is simply water with one extra atom of oxygen. This atom is unstable and tends to react with (oxidize) other biological molecules and damage them in the process. The control for hydrogen peroxide is the enzyme, catalase, one of the most efficient enzymes known.

Despite the purposeful attempt by the body to regulate the amounts of these two substances, some escape the surveillance system and react with each other, generating the most toxic and reactive of all ROTS, hydroxyl radical. This, as with superoxide, is a free radical and very damaging, particularly to the unsaturated lipids found in biological membranes.

All of these cytotoxic substances as well as a fourth (singlet oxygen) are specifically generated by certain white blood cells (WBC) when substances or particles (including bacteria and viruses) foreign to the body contact these white cells.

Since all members of the ROTS family of oxidizing agents are toxic to cells, those WBC capable of generating them are releasing these substances for the purpose of killing or damaging the invading organisms. Like many other biological systems, the process is not perfect and backfires, resulting in damage to the body as well as to the organism that is to be destroyed. Interestingly, the WBC capable of generating an attack on foreign organisms through ROTS production are those very cells that are also capable of phagocytosis, the process by which the killed organisms are taken up and digested.[7]

In every degenerative disease studied there is found the presence of excessive ROTS. Some of these degenerative diseases and other pathological states that are sources of ROTS are shown in chart 5.

We shall see in the sections that follow that the generation of ROTS by WBC sometimes works against the body by creating side effects that may, in the worst case, prove fatal. To know of these causative mechanisms and provide a remedy gives the physician an advantage in the management of what may otherwise lead to a disastrous situation.

Endotoxin as a Causative Factor in Disseminated Intravascular Coagulation

Almost as soon as it was recognized that bacteria are the cause of many human pathologies it was also learned that the toxic substances related to these bacteria are, in many cases, endotoxins.

The term endotoxin refers to a family of related substances, having certain characteristics in common but differing from one bacterial species (or strain) to another. Endotoxins are lipopolysaccharides (LPS), meaning a combination of lipids (fat) and polysaccharides (chains of sugars). From this definition alone, it may be seen that such substances form a widely diversified group simply because of (1) the number of long-chain fatty acids comprising lipids, (2) the number of individual sugars as well as their modes ➤

Historical Background of HLB Blood Test
(Free Radical Footprint)

The empirical evidence for the morphological changes in blood drops was first observed and reported in the 1940's by H.L. Bolen[1] at Massachusetts General Hospital. At that time the mechanisms were not understood and Bolen mistakenly attributed all the morphological changes to cancer. B.H. White *et al.*[2] in the 1950s empirically observed that other metabolic diseases could induce some of the same blood changes. Several physicians throughout the world continued work with similar blood tests (unreported), adding more empirical evidence. Among these were Heitan and Legard in France and Weiss in the United States. The Bradford Research Institute began research on the test in 1979 and launched the International HLB (Heitan, Legard, Bradford)-Metabolic Research Project to study, describe and explain the various morphologial changes. Bradford[3] developed the protocols and delineated the relationship between reactive oxygen toxic species (ROTS) and morphological blood changes in a wide range of metabolic dysfunctions (The Bradford-Allen Effect). (Free Radical Footprint)

1. *Journal of Laboratory and Clinical Medicine* 27 1522 (1942), Bolen, HL, The Blood Pattern as a Clue to the Diagnosis of Malignant Disease.
2. *American Journal of Surgery* 84 356 (1952), White, BH, et al., The Bolen Test for Cancer.
3. *Oxidology. The Study of Reactive Oxygen Toxic Species (ROTS) and their Metabolism in Health and Disease.* (The ROTS Theory of Degenerative Disease and the HLB Blood Test), Bradford, RW, et al., 1985. The Robert W. Bradford Foundation, Chula Vista, California.

➤

of linkage to one another, (3) the branching of sugar chains, and (4) the number of possible arrangements of all of these units into a single endotoxin molecule. Endotoxins also contain protein, further compounding the structural diversity.[8]

The purpose of endotoxin to the bacterium that produces it is to act as a semipermeable membrane, limiting and regulating the nature of substances that may enter and provide nutrient for that organism.[8]

For this reason endotoxins reside solely on or near the surface (cell wall) and are shed into the surrounding medium only upon the death of the organism. This fact may well be an explanation for what has become known as the Herxheimer reaction in which a patient becomes worse following the administration of antibiotics or other form of treatment that kills the causative organism.[8]

The fact that endotoxins cause harm to humans and other animals is purely coincidental since their function is not related to causing illness in animals. The reverse, however, is not true since there is much evidence that animals are highly aware of and respond to the presence of endotoxin, this substance signaling the presence of an invader.

These considerations lead to the interesting conclusion, borne out by the fossil record, that bacteria existed long before animals. Stated another way animals are aware of bacteria and were developed in terms of them, but bacteria are not aware of animals since they appeared first in the developmental sequence.

Experiments with endotoxins conducted over many years indicate that the toxic effects stem from only the lipid portion of the intact molecule. Most if not all of the toxic effects of endotoxin may be attributed to this unit and is sometimes used exclusively in experiments rather than the intact molecule.[8]

Basically, the structure of an endotoxin is the lipid common to all forms, designated "lipid A," to which is attached the "core" polysaccharide, identical for large groups of bacteria. To the core polysaccharide is attached the "O-antigen," consisting of various lengths of polysaccharide chains which are chemically unique for each type of organism and LPS. These sugar chains provide endotoxin its specificity.[8]

One additional feature of lipid A is its phosphate content. Each phosphate group carries a negative charge and since lipid A is a rather large molecule, provides a negatively charged surface related to the activation of a specific blood clotting factor (XII), to be described in greater detail.

Endotoxins are produced only by gram negative bacteria. These are bacteria that do not undergo cell wall staining by the gram stain, developed early in the study of bacteria and containing iodine.[8]

The Significance of Tissue Factor in DIC

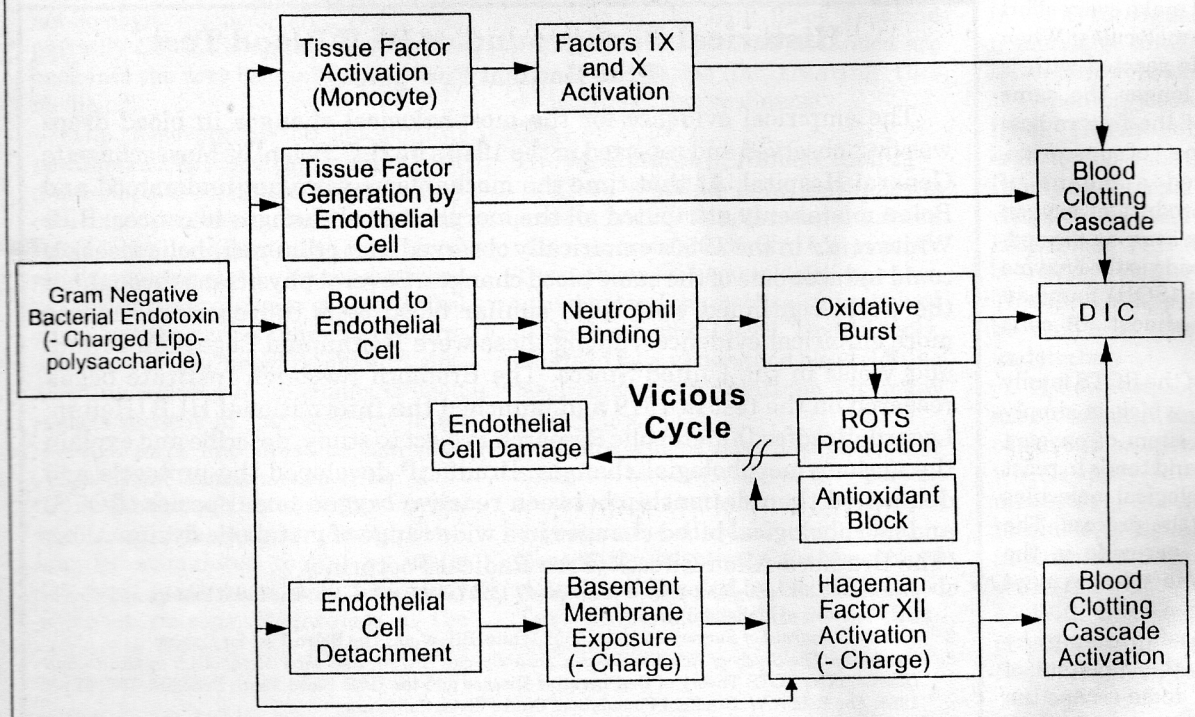
Of the utmost importance in an understanding of disseminated intravascular coagulation is a transmembrane lipoprotein having a molecular weight of 30,000 found on the surface of platelets, endothelial cells (those lining the blood vessels), leukocytes, monocytes and most cancer cells. This protein, formerly known as thromboplastin, has been renamed tissue factor (TF). It is not normally found on tissue cells but may be present in small amounts on the surface of certain blood cells such as platelets and monocytes.[9]

The gene for tissue factor is activated in wound healing and in other conditions. It is the primary *cell-bound* initiator of the blood coagulation cascade. By itself it is incapable of initiating clotting but becomes active when complexed with Factor VII or activated Factor VII (VIIa).[5]

Tissue factor has been described as the

Chart 1

Mechanism of Disseminated Intravascular Coagulation Generation from Endotoxin



receptor for Factor VII because of the close association between the two proteins as well as causing a shape change (conformational) in Factor VII, allowing it to attain activity.

The formation of the TF – Factor VII complex allows Factor VIIa (the activated form) to activate Factors IX and X, which initiates the blood clotting cascade and the formation of thrombin.[5]

Tissue factor plays a major role in several biochemical mechanisms leading to disseminated intravascular coagulation.

Disseminated Intravascular Coagulation Resulting from Endotoxin

An infection from gram negative bacteria results in the release of endotoxin which has a direct genetic effect on tissue factor gene expression in leukocytes. Contact of endotoxin with leukocytes activates proteins that bind to nucleotide (gene) sequences of DNA thereby activating the TF gene.[10] (See chart 1)

Tissue factor may also be expressed on the surface of endothelial cells grown in culture. In the absence of endotoxin no procoagulant activity could be detected. However, the addition of endotoxin from *E. coli* or *Salmonella enteritidis* resulted in procoagulant activity which reached a maximum in 4 to 6 hours and was dose-dependent.[11]

Endothelial cells in culture are damaged by endotoxin (or lipid A) sufficiently to attract polymorphonuclear leukocytes (PMN) which adhere to the cells. The oxidative injury resulting from bound PMNs could be blocked by free radical scavengers, showing that ROTS generated by PMNs was responsible for the damage.[12]

A single intravenous injection of *E. coli* endotoxin to experimental animals resulted in circulating endothelial cells within 5 minutes.[8] In other experiments with *E. coli* endotoxin, detachment of endothelial cells from the basement membrane was noted.[13]

The simple removal of endothelial cells from the inner lining of blood vessels has dire consequences from two standpoints. Endothelial cells form the inner surface of blood vessels which are always in contact with blood. The surface of these cells contacting blood are covered with a specific prostaglandin known as prostacyclin or PGI2 (prostaglandin I2). If for any reason blood contacts a surface not covered with PGI2, it will clot. Such surfaces devoid of this prostaglandin are formed whenever a cut is made, thereby exposing a surface not covered with PGI2. Also, an abrasion or injury may also expose a surface on which PGI2 is lacking.[14]

With the removal of endothelial cells by endotoxin, a surface is created devoid of PGI2, leading to blood clotting. But, there is yet another reason why blood clotting may be intiated by the removal of endothelial cells. Beneath these cells is the basement membrane, a thin continuous net of specialized extracellular matrix composed mainly of collagen, proteoglycans (sulfonated) and laminin.[15] The presence of sulfonated polysaccharides in the proteoglycans brings a negatively charged surface into direct contact with blood. The significance of this in relation to blood clotting is described below.

Blood Clotting from Contacting a Negatively Charged Surface

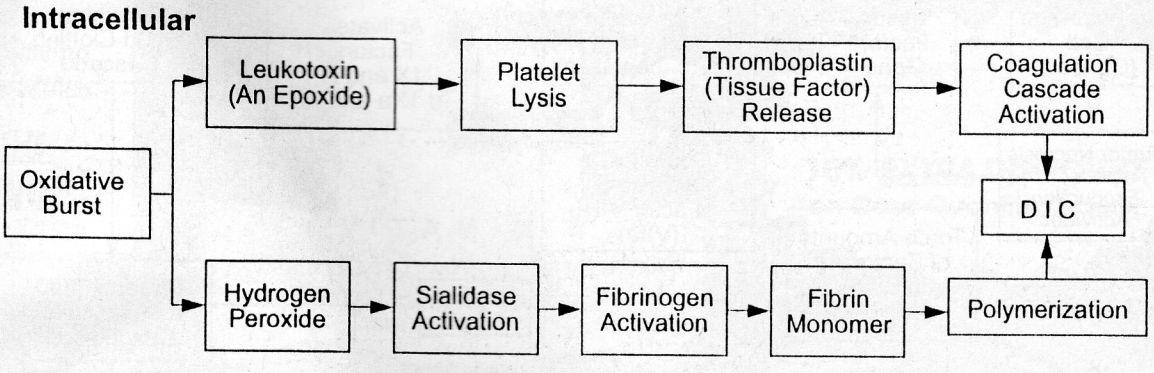
Thirty years ago (1964) it was discovered that blood will clot from simply contacting a negatively charged surface.[5] Previous to this, it was believed that the blood clotting process represented a cascade of enzyme activity in which one factor acting as an enzyme activated the next enzyme, and so on. The discovery that blood could be clotted simply by contacting a negatively charged surface ruled out the enzyme mechanisms. Only some of the known blood clotting factors have been shown to be enzymes.[5]

As a result of this surprising discovery, detailed research was conducted in an attempt to learn the mechanism. In some of the experiments the negatively charged surfaces of selected finely divided inorganic crystals including aluminum oxide, barium sulfate, jeweler's rouge, quartz and titanium oxide, were considered.[16]

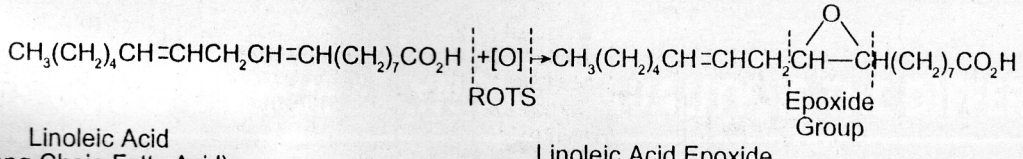
➤

Chart 2

Disseminated Intravascular Coagulation Resulting from a Phagocytic Oxidative Burst



Formation of Leukotoxin by ROTS



Linoleic Acid (Long Chain Fatty Acid)

Linoleic Acid Epoxide

If the negatively charged particles were smaller than the protein clotting factor itself, activation was minimal. Or if the concentration of clotting factor was too great, there was little or no activation. Both of these observations indicated that the activation process represented an electrostatic attraction between the negatively charged surface and positive charges on the clotting factor. This provided a clue that the clotting factor was a "basic" protein, that is, positively charged.[16]

The clotting factor that was eventually shown to be responsible for initiating blood clotting when whole blood contacted negatively charged surfaces was Factor XII, otherwise known as the Hageman factor. This is a positively charged protein, migrating in an electric field (electrophoresis) toward the anode.[16]

It is believed that Factor XII is normally in the shape of a hairpin which binds to the charged surface at the bend. Electrostatic attraction forces the two arms to lie flat on the surface, thereby exposing the inner faces and activating the molecule. Because of crowding or too small a surface Factor XII is not activated.[16] The highly phosphorylated lipid A of endotoxin (also negatively charged) activates Factor XII.[8]

Activation of Factor XII allows the activation of Factor XI which then activates Factor IX. Thus, the blood clotting cascade continues to the formation of fibrin the normal manner.[5]

As we shall see below, the activation of Factor XII through contact with a negatively charged surface (basement membrane, endotoxin, lipid A, cancer cell surfaces, etc.) provides a basic mechanism whereby such widely diverse areas as bacterial infection (sepsis), cancer and athersclerosis (see Chart 9) are interrelated.

The removal of endothelial cells by endotoxin exposes the negatively charged basement membrane (sulfonated polysaccharides) which in turn activates Factor XII. Activated Factor XII then activates Factors XI and IX, thereby initiating the blood clotting cascade.[5]

To summarize, endotoxin resulting from infection by gram negative bacteria has a multifactorial action with all roads leading to disseminated intravascular coagulation.

1. Activation of tissue factor gene in leukocytes, activation of Factors VII, IX and X resulting in the blood clotting cascade.
2. Activation of tissue factor gene in endothelial cells leading to Factor VII activation and the blood clotting cascade.
3. Endotoxin bound to endothelial cells resulting in neutrophil attraction and the generation of ROTS which, in turn, damages the membrane and attracts more leukocytes. Antioxidants block this vicious cycle.
4. The removal of endothelial cells exposes the negatively charged basement membrane leading to the activation of Factor XII (Hagemann) and initiation of the blood clotting cascade.
5. Lipid A component of endotoxin, being negatively charged, activates Factor XII and the blood clotting cascade.

Disseminated Intravascular Coagulation Resulting from ROTS

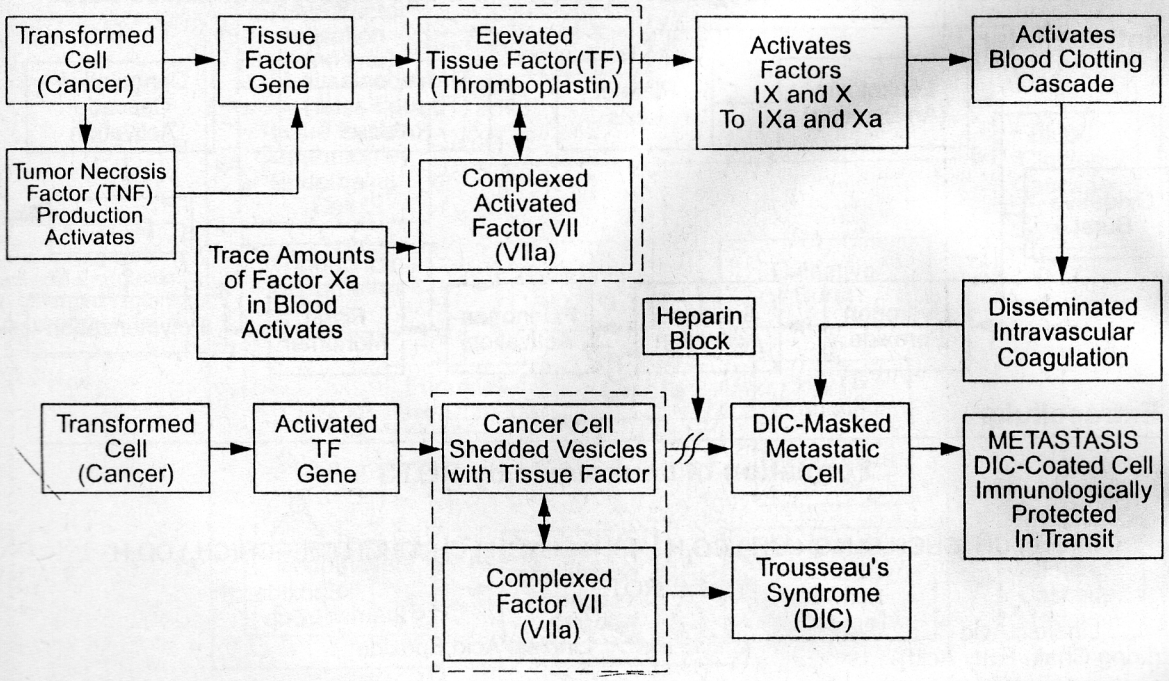
When certain white blood cells (leukocytes and others) are stimulated by the presence of particles (including bacteria, viruses, etc.), the result is the engulfment of the particles (phagocytosis) by the white cells (See chart 2).

White cells engaged in phagocytosis treat the particles as invading organisms to be killed. As a result, the white cells generate cytotoxic ROTS in an effort to kill them. The generation of ROTS by white cells during phagocytosis is referred to as an "oxidative burst."

One of the long-chain fatty acids present in the white cell is linoleic acid, having 18-carbons and 2 unsaturations. Unsaturated fatty acids are highly susceptible to oxidative attack and ordinarily lead to lipid peroxides. Subjected to ➤

Chart 3

Disseminated Intravascular Coagulation Resulting from Cancer



oxidation by ROTS generated by the leukocyte, linoleic acid binds a single oxygen atom thereby forming an epoxide at the first unsaturation.[17]

Subsequent research revealed that this compound, named leukotoxin, was highly toxic to cells and led to platelet lysis, thereby releasing tissue factor and initiating DIC.[17]

This sequence of biochemical events represents yet another mechanism, independent of endotoxin, whereby bacterial infection leads to disseminated intravascular coagulation.

Yet another mechanism leading to DIC from an oxidative burst from WBC and independent of endotoxin is the activation of the enzyme sialidase by hydrogen peroxide, a member of the ROTS family of oxidants.[18] Sialidase (neuraminidase) is an enzyme capable of removing a special sugar, sialic acid, from the terminal ends of polysaccharides, where it is always found. Many polysaccharides are not functional when sialic acid has been removed. The loss of sialic acid from red blood cells represents the aging of the cell and marks it for destruction by the liver.[20]

The activation of fibrinogen (the inactive precursor of fibrin) involves the removal of certain sialic acid residues as well as the release of two peptides termed peptide A and peptide B. Thus, the removal of sialic acid is a necessary requirement for the activation of fibrinogen to fibrin monomer, the unit of the fibrin chain.[19]

The enzyme sialidase is activated or stimulated by hydrogen peroxide, one of the products of an oxidative burst possibly arising from the phagocytosis of invading bacteria.[18] As a result of the phagocytosis of bacteria (either gram negative or gram positive), fibrinogen may be activated by sialidase, resulting in disseminated intravascular coagulation.

Thus, an oxidative burst, commonly resulting from the phagocytosis of bacteria, may result in DIC through the generation of fibrin monomer from sialidase activation by hydrogen peroxide.

The formation of leukotoxin, an epoxide of linoleic acid, by leukocytes results in the lysis of platelets, thereby releasing blood clotting factors and the formation of DIC.

Disseminated Intravascular Coagulation Resulting from Cancer

Many cancers have the ability to cause the production (in macrophages) of the lymphokine, cachectin (tumor necrosis factor or TNF). This protein has been shown to activate the gene for tissue factor (TF) in some cancer cells. Activation of the TF gene leads to increased expression of TF in the surface membrane of cancer cells.[21,22] (See chart 3)

Factor VII (activated to VIIa by a trace amount of Factor Xa in the blood) complexes with TF and undergoes a conformational change (change in shape) allowing the activation of Factors IX and X (to IXa and Xa). Factor Xa continues the blood clotting cascade until fibrinogen is activated, leading to fibrin formation.[5]

The ruffled nature of the surface of many cancer cells results in the formation of small projections in the plasma membrane which pinch off and release vesicles containing TF complexed with Factor VII.[22] The presence of excessive amounts of TF/Factor VII complexes on the surface of cancer cells allows the formation of a fibrin net around the cell. As a result of contact inhibition, cancer cells have the capability of leaving the primary tumor and becoming free-floating in the circulation. Cancer cells which have become free and enter tissue cells may lodge in other organs and begin multiplying. These metastasizing cells, because of the surrounding fibrin net, are thereby protected from attack by the immune system while in transit.[23,24]

The blockage of fibrin net formation by an anticoagulant such as heparin allow metastasizing cells to be attacked by natural killer (NK) cells and other immune cells and destroyed before reaching their targets.

To summarize, cancer cells, through activated macrophages, cause production of tumor necrosis factor (cachectin) which leads to the activation of the tissue factor gene of the cancer cell. This then leads to increased tissue factor production on the cancer cell surface and the resulting TF/Factor VII complex, capable of initiating the formation of fibrin around individual cancer cells. The protection afforded by the fibrin net prevents an attack by cytotoxic immune cells.

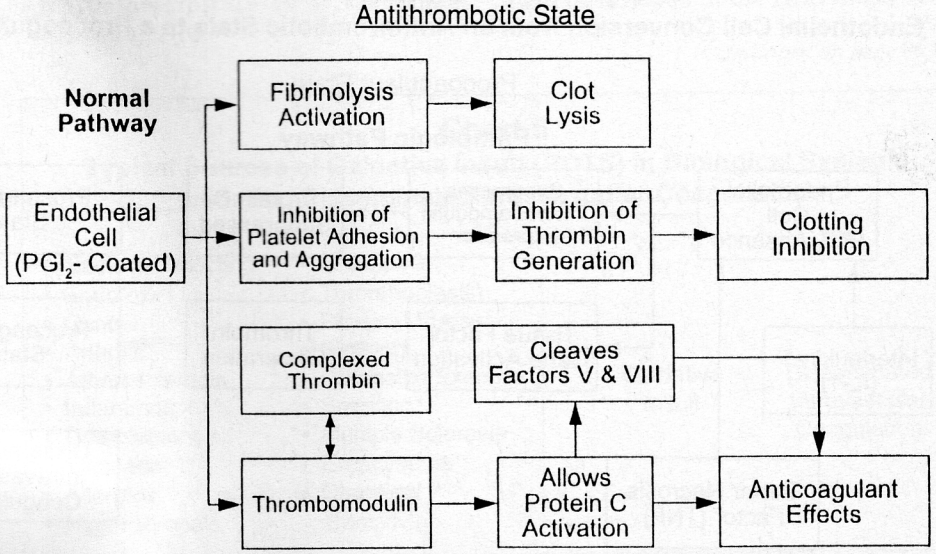
Conversion of Endothelial Cells From an Antithrombotic State to a Procoagulant State

As previously indicated, endothelial cells line the blood vessels and contain PGI2 (prostacyclin) on the surfaces in contact with blood to prevent blood clotting.[14]

➤

Chart 4a

Endothelial Cell Conversion from an Antithrombotic State to a Procoagulant State



Normal resting (unstimulated) endothelial cells show antithrombotic activity by the inhibition of platelet adhesion and aggregation, the inhibition of thrombin generation and the activation of the fibrinolytic system leading to clot lysis.[25]

Also on the surface of endothelial cells is a protein called thrombomodulin, which acts as a receptor for thrombin. When bound to thrombomodulin, thrombin has the ability to activate protein C. Activated protein C then catalyzes proteolytic cleavage of Factors Va and VIIIa that destroy their activity in blood clotting. In this manner thrombin, which normally activates fibrinogen resulting in fibrin (clotted blood), is made to destroy certain blood clotting factors and inhibit the clotting process.[5,26] (See chart 4a)

On the other side of the coin, this same endothelial cell which is capable of functioning as an antithrombotic agent becomes a procoagulant agent when acted on by certain cytokines such as interleukin-1 or tumor necrosis factor (TNF or cachectin). The activated endothelial cell expresses tissue factor (TF) on is surface as a result of TF gene activation. This leads to the production of thrombin as a result of triggering the blood clotting cascade.[25] (See chart 4b)

TNF has been shown to both induce tissue factor gene expression and to suppress transcription of the thrombomodulin gene in endothelial cells.[5,26] Many cytokines also stimulate adhesion of leukocytes to endothelial cells resulting in damage by ROTS as described previously.[25]

The Detection of Disseminated Intravascular Coagulation

The HLB Blood Test

The detection of disseminated intravascular coagulation (DIC) by means of the HLB Blood Test is a natural consequence of the nature of the test itself. A small amount of blood expressed from a fingertip is contacted with a clean microscope slide in a series of drops which are allowed to dry in a normal manner. (See chart 6)

During this process, blood is allowed to clot and be revealed by microscopic observation. The pattern seen in healthy persons is essentially the same, a dense mat of red areas interconnected by dark, irregular lines, completely filling the area of the drop.

The clotting pattern seen in one who is under oxidative stress, allergies, stress, psychological disturbances or other pathologies exhibits a variety of patterns characterized by various deviations from normal but with one striking abnormality common to all, that of certain areas in which the fibrin net/red blood cell conglomerate is missing. The extent and shape of the missing or clear areas is indicative of the particular pathologies affecting the individual. This observation is borne out by having the patient undergo therapy appropriate for the pathology or pathologies from which the patient is suffering. With successful treatment it becomes obvious from repeated testing with the HLB that the abnormal blood clotting pattern is returning to that of a normal person.

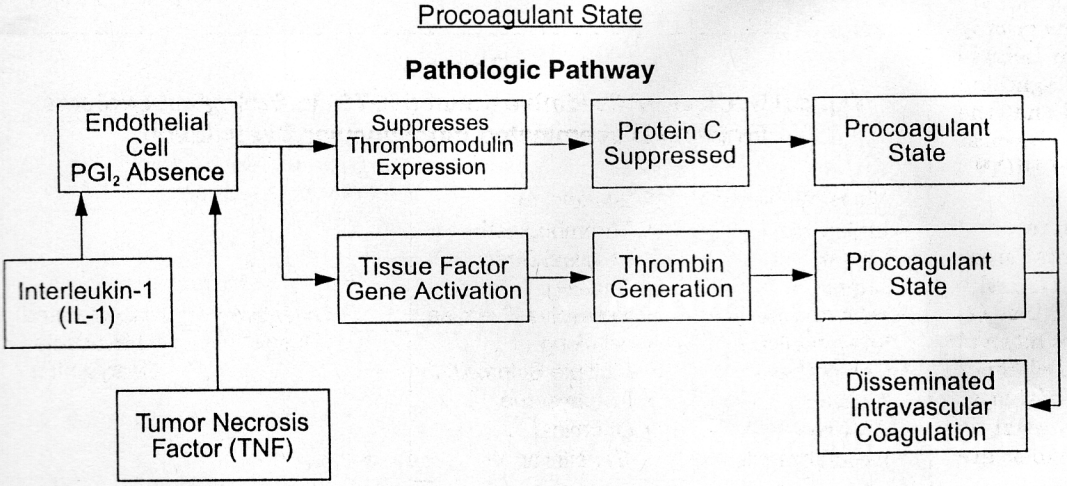
These changes or deviations from normal with pathology and treatment of that pathology are to be expected because a disease state is reflected by changes in blood composition and clotting ability as described above. The recognized hyper- and hypocoaguable states seen in various diseases as well as the generation of excessive ROTS and the inability to destroy them as seen in all degenerative diseases leads to the inevitable conclusion that these processes will beyond doubt lead to a modification of the normal blood clotting process and the development of an abnormal blood clotting pattern as seen in the HLB Blood Test.

DIC is characterized by the abnormal presence in the blood of fibrin, clotted blood, in a soluble form. When allowed to clot, blood containing such an abnormal artifact will exhibit distortions of that normally seen. In addition, the abnormal presence in the blood of soluble fragments of the extracellular matrix and soluble fibronectin will create an abnormal blood clotting pattern as well (to be described more fully in chart 7).

Because of its very nature the HLB Blood Test is amply suited to measure the presence in the blood of abnormal substances and clotting factors.

Chart 4b

Endothelial Cell Conversion from an Antithrombotic State to a Procoagulant State



The Sonoclot Coagulation Analyzer

The Sonoclot Coagulation Analyzer provides a rate-reaction record of fibrin and clot formation with platelet interaction. An axially vibrating probe is immersed to a controlled depth in a 0.4 ml. sample of blood. The viscous drag imposed upon the probe by the fluid is sensed by the transducer. The electronic circuitry quantifies the drag as a change in electrical output. The signal is transmitted to a strip chart recorder which

provides a representation of the entire clot formation, clot contraction and clot lysis process.

The analyzer is extemely sensitive to minute changes in viscoelasticity and records fibril formation at a very early stage.

The Sonoclot has been evaluated scientifically and shown to provide an accurate measurement of the clotting process.[27,28]

One application has been the development of a test to distinguish nonadvanced breast cancer from those that are benign. The rationale for the test is the hypercoaguable state seen in cancer patients (Trousseau's Syndrome), resulting from the generation of tissue factor by monocytes (leukocytes).[29]

Test for Soluble Fibrin

Basically, disseminated intravascular coagulation (DIC) may be considered as a two-step process. In the first, fibrinogen which is always present in the blood, is activated by any of several possible mechanisms. This activation leads to the formation of fibrin monomer and its automatic polymerization (chain formation) to soluble fibrin. This is not apparent unless the blood is allowed to clot (as is the HLB Blood Test).[30,31]

The second stage is the precipitation and deposition of soluble fibrin by several other mechanisms. One of these is the formation of cross-links through the action of Factor XIII and complexing with soluble fibronectin. Another such mechanism may be poor circulation in an organ already blocked by deposited fibrin. The deposition of precipitated fibrin may be detected microscopically in tissue sections and diagnosed as DIC.[32]

Because soluble fibrin is not readily detected, a chemical test for soluble fibrin is of immense value in diagnosing DIC. Research has indicated that the detection of soluble fibrin may be most useful in the early diagnosis of DIC.[33]

Fibrin Degradation Products

There are three fundamental areas related to blood clotting: (1) the prevention of blood from clotting, (2) the clotting or blood, and (3) the removal of clotted blood once it has formed. Since the presence of clotted blood, either at the macro level of micro level is damaging to the body, enzymes are present that are capable of removing (lysing) clotted blood.

The enzyme plasminogen is always present in the blood but is inactive as a proteolytic agent. Plasmogen activator converts inactive plasminogen to active plasmin, enabling it to degrade and solubilize fibrin. This degradation is not specific for fibrin, however, and may apply to other proteins as well.

When fibrin is degraded (fibrinolysis), there are more products formed than simply fibrin monomer. Commercial kits are available for the analysis of fibrin degradation. The result of this test is an indirect measure of the presence of DIC.[34]

Protamine Sulfate

Protamine is an arginine-rich protein which is found wrapped around the DNA of fish eggs and corresponds to the histones of higher animals. Since protamine is rich in the basic amino acids (arginine and lysine) it is positively charged and normally complex with the negatively charged phosphate groups of DNA. When isolated, the phosphate is replaced by sulfate thereby forming the salt, protamine sulfate.

A test has been developed which indicates fibrin strands and fibrin degradation products. The test is conducted in a test tube with fibrin monomer complexes forming early and the polymerization of fibrin degradation products forming later.[35]

Ethanol Gelation

The addition of ethanol to a solution containing fibrin monomer, resulting as a degradation product of fibrin, forms a white precipitate in a test tube, indicating DIC.[36]

Treatment of DIC

Heparin

Since disseminated intravascular coagulation is a disorder involving the abnormal clotting of blood, the most obvious treatment would be an anticoagulant. The most commonly used anticoagulant is heparin, a polysaccharide found normally in mast cells. The use of heparin may lead to a hypocoaguable state accentuated by the depletion of clotting factors resulting from DIC.[37]

Antioxidants

The use of antioxidants in the treatment (or prevention) of DIC is indicated at many points in the causative pathways. The damage to endothelial cells by endotoxin results in leukocyte binding and subsequent release of ROTS. Antioxidants will assist in preventing further damage to endothelial cells.

An oxidative burst resulting from phagocytosis arising from bacterial infection releases hydrogen peroxide which activates sialidase.[18] This enzyme is then able to activate fibrinogen leading to fibrin (DIC).

A third value in the use of antioxidants lies in counteracting oxidative stress. The result of such stress is the generation of hydroxyl radical which inactivates alpha1-antitrypsin inhibitor responsible, in a series of steps, for the breakdown of the extracellular matrix, to be more fully described in chart 7.

Although not directly related to DIC, this value in the use of antioxidants will be revealed in the HLB Blood Test

continued on page 86 ➤

Chart 5

Typical Sources of Oxidative Insult (ROTS) in Biological Systems Initiating Disseminated Intravascular Coagulation

- Bacterial Infection
- Endotoxin
- Cancer
- Arthritis
- Atherosclerosis
- Inflammation
- Complement Cascade
- Allergies
- Hyperadrenals
- Leukotoxin
- Thromboplastin
- Physical Stress
- Bruising
- Excessive Exercise
- Smoking
- Multiple Sclerosis
- Emphysema
- Chemical Sensitivity

→ Oxidative Insult → Disseminated Intravascular Coagulation

as an indicator of an effective therapeutic approach as related to the causative factors of DIC.

Accelerated Charge Neutralization (ACN)

Accelerated Charge Neutralization or ACN represents the reversal of the normal negative charge of most malignant tumors by an electronic device. As early as 1949 it was realized that the cervixes of patients having malignancies were negative in relation to other tissue. Eighty-one percent of those examined had cancer in the cervix.[38] The surfaces of tumors carry a negative charge greater than that of normal tissues.

Other experiments with tumors implanted in animals indicated that with charge reversal there was a corresponding inhibition of tumor growth.[39]

As indicated previously, contact of blood with a negatively charged surface initiates blood clotting (Factor XII activation). The formation of a fibrin net around the tumor acts as a shield in protecting it against the immune system. In addition, the formation of fibrin around individual cancer cells gives them protection against cytotoxic cells of the immune system during migration to another organ (metastasis).[23,24]

For these reasons and possibly others unknown there is an advantage in cancer therapy to reverse the charge of a tumor, making it more positive relative to its surroundings.

This may be accomplished through the use of an electronic instrument that applies a reversed voltage to the tumor by means of an electrode.[40] (see chart 8)

Saruplase

As described previously, plasminogen is the inactive precursor of plasmin, the proteolytic enzyme which degrades and solubilizes fibrin. Before becoming active as a degradative enzyme, plasminogen must be activated to plasmin. The gene for one of the naturally occurring plasminogen activators (full length single chain urokinase type) has been isolated and inserted into the common intestinal bacteria, *E. coli*. These bacteria are then cultured and the desired protein isolated and purified.

The advantage of this product over other previously employed activators is that thrombolysis occurs with little or no fibrinogenolysis (degradation and activation of fibronogen). This protein represents a safe way to treat DIC without previous complications.[41]

All-Trans Retinoic Acid

As previously described (chart 4b), thromobomodulin normally expressed on the surface of endothelial cells bind thrombin and, when bound, activates protein-C which cleaves and inactivates Factors V and VIII, required clotting factors. Taken together, the up-regulation of the thrombomodulin gene as well as the downregulation of the tissue factor gene would discourage the development of DIC. Also mentioned previously was the up-regulation of the tissue factor gene by tumor necrosis factor (TNF).

Retinoic acid, a derivative of vitamin A, is chemically a long-chain hydrocarbon containing several unsaturatious (carbon-carbon double bonds). An unsaturation can be in either of two forms, cis or trans. In the cis configuration both of the adjoining groups lie on the same side of the double bond while in the trans configuration the adjoining groups lie on opposite sides of the double bond. If all of the unsaturations in a molecule are in the trans configuration, the molecule is called "all-trans."

Retinoic acid in the all-trans isomeric form has been shown to block the up-regulation of the tissue factor gene as well as block the downregulation of the thrombomodulin gene induced by TNF.

This has been demonstrated only for acute promyelocytic leukemia but may be applicable to other forms of cancer as well. Patients with this disease are at high risk for the development of DIC and are improved by the use of all-trans retinoic acid.[21]

Bacteriostatic Agents

Antibiotics which kill gram negative bacteria will lower the formation of endotoxin and alleviate the adverse effects previously shown in chart 1.

Modified HLB Blood Test from Solubilized Extracellular Matrix

Clinical experience indicates that the HLB blood test is a measure of oxidative stress and, concurrently, of various degenerative diseases associated with oxidative processes occurring in patients having these diseases.

There is now a clearer picture of the biochemical rationale for the correlation of an abnormal blood clotting pattern with the presence of degenerative diseases. The connecting links between disease states and the distorted patterns of clotted blood as seen in the HLB blood test have been more clearly delineated.

The abnormal clotting pattern seen in the HLB Blood Test arising from pathological states is related to the presence in the blood of water soluble fragments of those substances that lie

➤

Chart 6

The Detection and Treatment of Disseminated Intravascular Coagulation (DIC)

Detection of DIC

- HLB Blood Test (Microscopy)
- Sonoclot (Viscosity Changes)
- Soluble Fibrin (Blood Chemistry)
- Fibrin Degradation Products (Blood Chemistry)
- Protamine Sulfate (Fibrin Complexing)
- Ethanol (Precipitation)

Treatment of DIC

- Heparin (Anticoagulant)
- Antioxidants (Free Radical Scavengers)
- Accelerated Charge Neutralization (Tissue Charge) (ACN)
- Saruplase (Fibrinolysis)
- All-Trans Retinoic Acid (Gene Suppression)
- Bacteriostatic Agents (Endotoxin)

between cells and hold them together, namely, the extracellular matrix (EM). Several enzymes are capable of degrading the components of the EM but are normally held in check by inhibitors. The inhibitors may be inactivated through oxidation by ROTS thereby freeing the degradative enzymes to attack the EM. The degradation of the EM releases water soluble fragments which modify the normal blood clotting pattern of the HLB blood test. (See chart 7)

Still another mechanism of clotted blood modification, also traced to the presence of ROTS, is the activation of the enzyme (sialidase) capable of removing sialic acid from erythrocytes (RBC), glycoproteins and blood fibrinogen.[18] The removal of sialic acid from fibrinogen allows it to form long-chain water soluble molecules of fibrin which also modify the pattern of the HLB blood test.

These mechanisms of altered blood clotting will be described in greater detail below.

Extracellular Matrix Degradation

The EM is a three-dimensional gel binding cells together, comprised of four or more major constituents, collagen (protein), hyaluronic acid (polysaccharide), proteoglycans (protein/polysaccharide) and fibronectin. Also included in this list are the glycosaminoglycans and elastin.[42]

One of the proteolytic enzymes (capable of degrading protein) is trypsin, normally inactivated by alpha1-antitrypsin inhibitor, also a protein. The active portion of the inhibitor contains the amino acid, methionine, which includes a C-S-C linkage. When oxidized by hydroxyl radical (one of the ROTS family of oxidants), the central sulfur atom acquires one or two oxygen atoms, forming the sulfone or sulfoxide of methionine, respectively. The oxidation of methionine inactivates the inhibitor, thereby allowing trypsin to degrade the protein, collagen.[43]

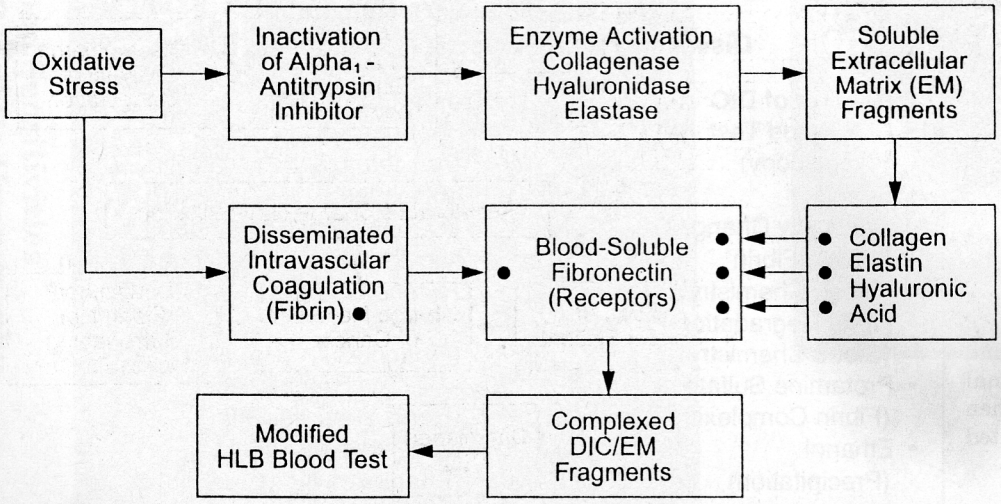
Thus, the activity of ROTS results in short-chain, water soluble collagen fragments in the blood of those suffering from various degenerative diseases. In every degenerative disease studied there has been found evidence for an abnormal increase in ROTS activity. Additional ROTS activity leads to the degradation of proteoglycans, hyaluronic acid and elastin with the formation of water soluble fragments of these normally insoluble EM components. The presence of fragments of the EM in blood is therefore an indication of EM degradation by ROTS as found in degenerative diseases. Also present is the unit of fibrin (fibrin monomer) which has been found in the blood of patients suffering from collagen disease.[44]

Fibronectin is a molecule having several binding sites for various long-chain biomolecules, for example collagen and heparin (a sulfonated polysaccharide). As such, it functions as a cellular glue, binding cells together as well as components of the EM.[42]

A form of fibronectin is found free in the blood and enters into the formation of a blood clot through the action of factor XIII, a member of the blood clotting cascade.[44] This form of fibronectin binds to fibrin, the major protein comprising clotted blood.[42] The blood fibronectin is not only elevated[45] in such degenerative diseases as arthritis and emphysema (collagen diseases) but *fragmented* by hydroxyl radical.[46] To fragment the molecule binding together various long-chain biosubstances (polymers) will certainly modify the blood clotting pattern as seen in the HLB blood test.

The presence in the blood of short-chain, water soluble fragments of the EM bound together by fibronectin forms a three-dimensional interconnected network or gel superimposed on and intermingled with the normal blood clotting units (fibrin and components of the blood clotting cascade). Since fibronectin binds to both fibrin and collagen,[42] the two polymeric networks are superimposed and result in a modification of the normal clotting pattern. Exactly how this normal pattern is modified will depend on the nature of the collagen abnormally present, the nature and extent of hyaluronate present and the degree to which fibronectin itself has been fragmented by ROTS. Thus, it is easily seen that there are endless forms the pattern of clotted blood may take, depending on the nature of the degenerative disease that produced the modifying substances. In this manner the HLB blood test reveals not only the presence of oxidative stress but indicates as well the nature of the disease which has resulted from that stress.

Chart 7
Modified HLB Blood Test
Complexing DIC with Solubilized Extracellular Matrix



Atherosclerosis Resulting from Bacterial Infection

It was indicated in the section, Disseminated Intravascular Coagulation Resulting from Endotoxin, that infection by gram negative bacteria because of the production of endotoxin by these

organisms, may lead to the removal of endothelial cells from the inner lining of blood vessels. The removal of endothelial cells exposes the negatively charged basement membrane, a thin layer of collagen and other extracellular matrix components including the proteoglycans. A part of the molecule comprising the latter group consists of a sulfonated polysaccharide carrying a negative charge in the sulfate group. (See chart 9)

As indicated previously, contact of blood with a negatively charged surface results in the activation of Factor XII (Hageman factor). This, in turn, activates Factor XI and so on through the blood clotting sequence to fibrin. The clotting of blood also involves the aggregation and clumping of platelets, thereby initiating on the inner lining of blood vessels the beginnings of atherosclerosis. The attraction of white cells leads to foam cell generation and the growth of atherosclerotic plaques.

This concept, that of the natural generation of fibrin resulting from blood contacting a negatively charged surface, has been expressed in relation to the kidney in explaining the deposition of fibrin in the urinary space of the glomerulus (extracapillary glomerulonephritis). In this disease the basement membrane is altered so as to present to the blood a negatively charged surface, leading to fibrin deposition.

Treatment with heparin was shown to decrease the severity of experimental glomerular disease. In addition, treatment with a plasminogen activator (plasmin dissolves fibrin) also resulted in improvement.[47]

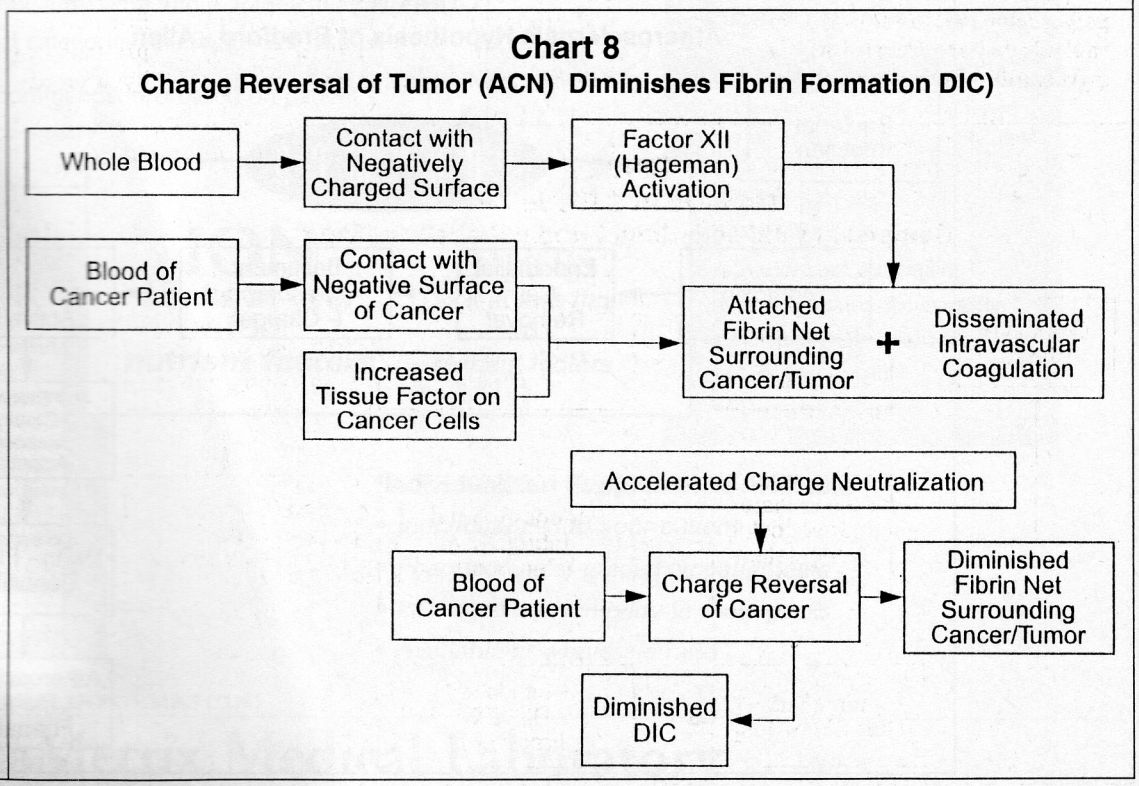
Since this mechanism has not, as far as is known by the authors, been presented as a possible cause of atherosclerosis, we are in this publication, setting forth the Atherosclerosis Hypothesis of Bradford-Allen.

If true, a possible mechanism of atherosclerosis generation as presented, leads to certain precautionary measures to be taken during and following a severe infection from gram negative bacteria. Since endotoxins are present during and following infection, particularly if antibiotics are used as a therapeutic measure, it would be advantageous to also include as a part of the therapy the use of (1) an anticoagulant such as heparin, (2) a plasminogen activator, and (3) fibrinolysis agents, for example saruplase.[41]

Correspondence:
Robert W. Bradford, D.Sc.
Robert W. Bradford Research Institute
1180 Walnut Avenue
Chula Vista, CA 91911
619-429-8200
800-243-7435

References

1. Jones, TW, Observations on some points in the anatomy, physiology, and pathology of the blood, *British Foreign Medical Review* 14, 585, (1842).
2. Trousseau, A, Phlegmasia alba dolens, *Cinique Medicle de l'Hotel - Dieu de Paris* 3 94 (1865).
3. Virchow, R, *Gesammelte Abhandlungen zur Wissenschaftlichen Medicin,* 1856, pg. 477.
4. Kitchens, CS, Concept of Hypercoagulability: A review of its development, clinical application, and recent progress, *Seminars in Thrombosis & Hemostasis* 11 293 (1985).
5. Rapaport, SI, Blood coagulation and its alterations in hemorrhagic and thrombotic disorders, *The Western Journal of Medicine* 158 153 (1993).
6. Hamilton, PJ, et al., Disseminated intravascular coagulation: A review, *Journal of Clinical Pathology* 31 609 (1978).
7. Bradford, RW, Allen, HW and Culbert, ML, Oxidology, The Study of Reactive Oxygen Toxic Species (ROTS) and Their Metabolism in Health and Disease, The Robert W. Bradford Foundation, Chula Vista, CA 91911, (1985).
8. Morrison, DC, et al., The effects of bacterial endotoxins on host mediation systems, *American Journal of Pathology* 93 526 (1978).
9. Hu, T, et al., Synthesis of tissue factor messenger RNA and procoagulant activity in breast cancer cells in response to serum stimulation, *Thrombosis Research* 72 155 (1993).
10. Mackman, N, et al., Lipopolysaccharide – mediated transcriptional activation of the human tissue factor gene in THP-1 monocytic cells requires both activator protein 1 and nuclear factor kappa B binding sites, *Journal of Experimental Medicine* 174 1517 (1991).
11. Colucci, M, et al., Cultured human endothelial cells generate tissue factor in response to endotoxin, *Journal of Clinical Investigation* 71 1893 (1983).
12. Yamada, O, et al., Deleterious effects of endotoxin on cultured endothelial cells: An in vitro model of vascular injury, *Inflammation* 5 115 (1981).
13. Cho, TH, et al., Effects of Escherichia coli endotoxin on structure and permeability of myocardial capillaries, *Acta Pathologia Japonica* 41 12 (1991).
14. Risberg, B, et al., Disseminated intravascular coagulation, *Acta Anaesthesiologica Scandinavica, Supplementum* 95 60 (1991).



15. Alberts, B, et al., editors, *Molecular Biology of the Cell, Second Edition,* Garland Publishing, Inc., New York, pg. 818, (1989).
16. Margolis, J, The interrelationship of coagulation of plasma and release of peptides, *Annals of the New York Academy of Sciences* 104 133 (1963).
17. Sugiyama, S, et al., The role of leukotoxin (9,10-epoxy-12-octadecenoate) in the genesis of coagulation abnormalities, *Life Sciences* 43 221 (1988).
18. Mueller, HE, et al., Increase of microbial neuraminidase activity by the hydrogen peroxide concentration, *Experientia* 23 397 (1972).
19. White, A, et al., editors, *Principles of Biochemistry,* McGraw-Hill Book Co., New York, pg. 648 (1964).
20. Aminoff, D, et al., Cell surface carbohydrate recognition and the viability of erythrocytes in circulation, *Progress in Clinical & Biological Research* 23 569 (1978).
21. Tallman, MS, et al., New insights into the pathogenesis of coagulation dysfunction in acute promyelocytic leukemia, *Leukemia & Lymphoma* 11 27 (1993).
22. Silberberg, M, et al., Identification of tissue factor in two human pancreatic cancer cell lines, *Cancer Research* 49 5443 (1989).
23. Grimstad, IA, et al., Thromboplastin release, but not content, correlates with spontaneous metastasis of cancer cells, *International Journal of Cancer* 41 427 (1988).
24. Gunji, Y, et al., Role of fibrin coagulation in protection of murine tumor cells from destruction by mytotoxic cells, *Cancer Research* 48 5216 (1988).
25. Beretz, A, et al., Modulation by cytokines of leukocyte-endothelical cell interactions. Implications for thrombosis, *Bioheology* 27 455 (1990).
26. Nachman, RL, et al., Hypercoagulable states, *Annals of Internal Medicine* 119 819 (1993).
27. Chandler, WL, et al., Evaluation of a new dynamic viscometer for measuring the viscosity of whole blood and plasma, *Clinical Chemistry* 32 505 (1986).
28. Saleem, A, et al., Viscoelastic measurement of clot formation: A new test of platelet function, *Annals of Clinical & Laboratory Science* 13 115 (1983).
29. Spillert, CR, et al., Altered Coagulability: An aid to selective breast biopsy, *Journal of the National Medical Association* 85 273 (1993).
30. Bowie, EJ, et al., The clinical pathology of intravascular coagulation, *Bibiotheca Haematologica* 49 217 (1983).
31. Muller-Berghaus, G, et al., The role of granulocytes in the activation of intravascular coagulation and the precipitation of soluble fibrin by endotoxin, *Blood* 45 631 (1975).
32. Bick, RL, Disseminated Intravascular Coagulation, *Hemoatology / Oncology Clinics of North America* 6 1259 (1993).
33. Bredbacka, S, et al., Laboratory methods for detecting disseminated intravascular coagulation (DIC): New aspects, *Acta Anaesthesiologica Scandinavica* 37 125 (1993).
34. Sigma Diagnostics, St. Louis, MO 63178.
35. Detection of intravascular coagulation by a serial-dilution protamine sulfate test, *Annals of Internal Medicine* 75 895 (1971).
36. Breen, FA, et al., Ethanol gelatin: A rapid screening test for intravascular coagulation, ibid., 69 1197 (1970).
37. Karmad, Dr, et al., Disseminated intravascular coagulation: A review with experience from an intensive care unit in India, *Journal of Postgraduate Medicine* 38 186 (1992).
38. Langman, L, et al., A technique to aid in the detection of malignancy of the female genital tract, *American Journal of Obstetrics & Gynecology* 57 274 (1949).
39. Humphrey, CE, et al., Biophysical approach toward tumor regression in mice, *Science* 130 388 (1959).
40. Bradford, RW, et al., Accelerated Charge Neutralization: Biological charges and their significance in health, disease and therapy, Bradford Research Institute, Chula Vista, CA 91911.
41. Ostermann, H, et al., Rate of fibrinogen breakdown related to coronary patency and bleeding complication in patients with thrombolysis in acute myocardial infarction – Results from the PRIMI trial, *European Heart Journal* 13 1225 (1992).
42. Hay, ED, ed, *Cell Biology of Extracellular Matrix,* Plenum Press, New York, (1981).
43. Carp, H, et al., In vitro suppression of serum elastase-inhibitory capacity by reactive oxygen species generated by phagocytosing polymorphonuclear leukocytes, *Journal of Clinical Investigation* 63 793 (1979).
44. Wilson, CL, The alternatively spliced V region contributes to the differential incorporation of plasma and cellular fibronectins into fibrin clots, *Journal of Cell Biology* 119 923(1992).
45. Kawamura, K, et al., Enzyme immunoassay of human plasma fibronectin in malignant, collagen and liver diseases, *Clinica Chimica Acta* 131 101 (1983).
46. Vissers, MC, et al., Oxidative damage to fibronectin. II. The effect of H2O2 and the hydroxyl radical, *Archives of Biochemistry & Biophysics* 285 357 (1991).
47. Roudeau, E, et al., Role of homeostasis in the formation of cresants in extracapillary gomerulonephritis, *Nephrolgie* 13 259 (1992).