Incorporation of Radiolabelled Fibrinogen into Venous Thrombi Induced in Dogs

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SUMMARY

The time course and locus of radiolabelled fibrinogen (RLF) incorporation into venous thrombi were studied in dogs. Incorporation of radiolabelled fibrinogen (RLF) into fresh venous thrombi induced in vivo was studied by serial autoradiography, well-counting and surface counting. Autoradiographs disclosed diffuse incorporation of RLF into thrombi formed before and after RLF injection. Well-counting data also reflected thrombus RLF accumulation in both instances, although this accumulation was discernible earlier in thrombi induced in the presence of circulating RLF. Thrombi induced prior to RLF injection were detectable by surface counting after six hours. These studies support the potential value of RLF in detecting human venous thrombosis but also point to the limitations of the technique as well as questions which require clarification.

Additional Indexing Words:
Surface counting Well-counting

PULMONARY EMBOLISM continues to be a major cause of potentially-preventable morbidity and mortality, particularly among hospitalized patients. Early detection of its most common precursor, venous thrombosis of the lower extremities, would permit prompt therapy and reduce the frequency of embolism. Unfortunately, the clinical diagnosis of venous thrombosis is neither specific nor reliable. In several series in which clinical diagnosis has been measured against phlebography or autopsy, venous thrombi in more than 50% of patients escaped clinical discovery. The unreliability of clinical diagnosis has emphasized the need for a simple, rapid and reliable means for detection of venous thrombosis. One potential candidate for fulfilling this need is the radiolabelled fibrinogen (RLF) method.

Since 1960, when Hobbs and Davies demonstrated that incorporation of RLF did permit detection of fresh venous thrombi in vivo by surface counting, there have been extensive positive clinical experiences with this technique in England. However, questions also have been raised concerning the use and interpretation of the method in human thromboembolism. These questions reflect the fact that virtually all of the experience with RLF has been in man, and human investigation places obvious constraints upon the observations which can be made. Particularly at issue are such unresolved fundamental questions as the time course and pattern of RLF incorporation into thrombi in vivo; the value of the technique in detecting venous thrombi formed prior to RLF injection; and the relationship between RLF incorporation into the thrombus and the ability to detect this thrombus by surface counting. This study was designed to provide information bearing upon these questions by defining: (1) the histologic and autoradiographic configurations of venous thrombi formed before and after the intravenous injection of RLF; and (2) the time course of radiolabel incorporation into venous thrombi formed before and after injection of 125I-fibrinogen, as assessed both by well-counting of harvested thrombi and by surface counting.

Methods

Conditioned mongrel dogs that weighed 20–30 kg were maintained on a standard diet except that Lugol’s solution (10 drops/day) was added to the drinking water the day prior to study and each day thereafter for 3 weeks to block the uptake of iodine by the thyroid. Highly-purified human fibrinogen was labelled with...
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Iodine or \(^{125}\)I by the method of McConahey and Dixon.\(^{13}\) Human serum albumin was labelled in the same manner.

Venous thrombi for all study phases except those involving surface counting were induced in vivo in these animals by the method of Wessler.\(^{14}\) A portion of the common femoral vein was exposed and ligatures placed proximally and distally. Within 90 sec after injection 30–60 cc of human serum or serum eluate,\(^{14}\) both ligatures were tied. After 30 min of total stasis, the distal ligature was removed and the proximal ligature loosened to allow blood flow over the resultant thrombus. In some animals, 40 \(\mu\)L of RLF was injected before thrombus formation. Thrombus was induced when count rates monitored over the heart had reached a stable plateau. In other animals, the RLF was injected 30 min after exposure of the thrombus to the circulating blood. Human, rather than canine, fibrinogen was used because it was readily available in highly-purified form\(^*\) and because the antigenic potential would not influence the results of these acute studies. Human serum or eluate was used because it has been as effective as canine products in inducing venous thrombosis.\(^{14}\)

\(^*\)This fibrinogen is 98% clottable with thrombin. It contains trace amounts of plasminogen.

In the surface counting studies, the procedure was modified to avoid incision over the site of the thrombus because incision led to accumulation of the RLF in the tissues. A #12 Swan-Ganz balloon catheter was passed from a foot vein to the femoral vein under fluoroscopic control. Serum or eluate was then injected intravenously and, within 90 sec, the balloon was inflated to occlude the vein totally. After 30 min, the balloon was partially deflated, exposing the thrombus that had formed to blood flow. RLF was injected 30 min later. In all animals, the presence of a thrombus was confirmed by phlebography.

All surface counting was performed with a Pittman ratemeter Model 235 Isotope Localizer (International Instrument Corporation). The counts were taken for two minute periods at 4 cm intervals from inguinal ligament to heel.

The volume of all thrombi submitted to well-counting was determined by water displacement, and the radioactivity was determined in a Packard autogamma 410A system. An equal volume of (clotted) blood which had been removed at the same time was handled identically and served as the control.

Thrombi removed for microscopic and autoradiographic studies were fixed in 10% neutral formalin, embedded in paraffin, cut in 4-8 micron sections and stained with hematoxylin and eosin. Some sections were

(A) Experimental thrombus removed 30 minutes after exposure to circulating blood. A thin layer of fibrin and formed elements (left) has been deposited. (B) Thrombus removed after 3 hours' exposure to circulating blood. Increased surface layering and infiltrations of polymorphonuclear leucocytes are seen. (Hematoxylin/eosin, \(\times 100\))

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coated with Kodak NTB autoradiographic stripping emulsion and developed in Kodak D-19 after 14 days' exposure.

Results

Routine Histology

Twenty thrombi induced by the ligature-serum method were removed from 12 dogs at intervals of 1/2 to 27 hours after exposure to the circulation. Staining with hematoxylin and eosin disclosed a characteristic layering of fibrin and formed elements on the surfaces of all thrombi examined. Thrombi removed 30 minutes after exposure showed small amounts of surface deposition (fig. 1A), the extent of which increased with time. By three hours, fibrin deposition was quite extensive and many polymorphonuclear leukocytes were present (fig. 1B). Beyond 6–12 hours, the age of the thrombus was difficult to assess. Some thrombi had resolved completely by 6–7 days.

Autoradiography

Fourteen animals were studied. In six, RLF was injected prior to thrombus formation; six were injected 30 minutes after the thrombus was exposed to circulating blood. In two, a thrombus was formed in one femoral vein prior to injection of $^{131}$I-albumin and a second thrombus was formed in the contralateral femoral vein one hour later. Thrombi were harvested at intervals of 1–24 hours after injection of radiolabelled material.

Thrombi formed after injection of RLF showed, as anticipated, diffuse radioactivity. In addition, having been exposed to the circulating fibrinogen, they showed radioactivity in the layers in which new fibrin had been laid down (fig. 2A). Thrombi formed before injection of radiolabel showed not only the anticipated deposition of radioactivity on their surfaces but also diffuse activity throughout their structures (fig. 2B). In thrombi formed both before and after RLF injection, these autoradiographic patterns were present in the earliest thrombi harvested and persisted.

Thrombi formed before or after injection of $^{131}$I-labelled albumin failed to show significant radioactivity either on their surfaces or within their substance.

Thrombus Radioactivity by Well-Counting

In six animals, RLF was injected prior to thrombus formation by the serum-ligature method and the thrombi were removed one to 217 hours after formation. Beyond 1/2 hours, radioactivity in the thrombus was consistently above that in the (clotted blood) control (fig. 3).

Figure 2

(A) Autoradiograph-photomicrograph of thrombus formed after injection of RLF. This thrombus had been exposed to circulation for 30 minutes. There is diffuse stippling (radioactivity) in both surface layer (above) and core of thrombus. Some polymorphonuclear leucocytes are evident. (X 200) (B) Thrombus formed before injection of RLF and exposed to circulation for two hours. Diffuse stippling (radioactivity) is present in both surface layers (left) and core of thrombus. (X 100)
In seven additional animals, the RLF was injected 30 minutes after exposure of the thrombus to blood flow. Thrombus/control differences in radioactivity were not consistent at six hours (fig. 4). However, at 12, 18, and 24 hours, thrombus radioactivity was consistently well above clotted blood control values (fig. 4).

When radiolabelled albumin was injected before or after thrombus formation, thrombus radioactivity was below clotted blood radioactivity during the first three hours (fig. 3) and at 6, 12, 18 and 36 hours.

**Thrombus Radioactivity by Surface Counting**

In 10 dogs, the Wessler method was modified by utilizing a balloon catheter to produce stasis, as described under Methods, and RLF was injected 30 minutes later. Counts over the leg containing the thrombus and over the contralateral leg were monitored every three hours. Small but unequivocal differences in radioactivity appeared consistently at six hours. These differences were accentuated with the passage of time (figs. 5, 6). At 22 hours all of these thrombi were removed, and all had significantly more radioactivity than clotted blood by well-counting.

**Additional Observations**

Serial surface count rates were performed in five dogs in which one femoral vein was ligated and in five in which one femoral vein was obstructed by a Swan-Ganz catheter. No consistent differences in radioactivity between control and experimental legs were observed during 24 hours. Edema (detectable by clinical exam) did not develop in the legs of any dogs.

**Discussion**

The concept of surface detection of intravascular thrombi after injection of a "thrombus specific" material is not new. Radiolabelled plasminogen,
streptokinase, antifibrinogen and other materials—in addition to fibrinogen—have been or are currently being explored for this purpose. This experience has indicated that three major criteria must be met by any material considered for this purpose: (1) the radiolabelled material must be specifically incorporated in the thrombus; (2) such incorporation must be sufficient to provide a significant difference between thrombus radioactivity and background radioactivity, significant meaning that the difference can be detected consistently by surface detectors; (3) it must be non-toxic.

**Thrombus Specificity**

Circulating RLF is inherently thrombus-specific for the "red" fibrin thrombi encountered in the venous system. Furthermore, it should be incorporated into fresh venous thrombi exposed to the circulating blood as the characteristic fibrin-formed element layers ("lines of Zahn") are laid down. Our microscopic observations confirm that the characteristic layering occurs in the thrombus model we employed, as it does in human venous thrombotic material. The autoradiographic observations show the expected diffuse radioactivity within thrombi formed in the presence of circulating RLF and the accretion of radioactivity in the surface layers. The absence of these findings with radiolabelled albumin suggests that fibrinogen accumulates specifically. Somewhat unexpectedly, there was also diffuse (as well as surface) radioactivity in thrombi formed prior to injection of RLF. This may reflect dissolution-reformation balance or actual interchange of thrombus fibrinogen with circulating fibrinogen. Radioactivity observed in polymorphonuclear leukocytes within the thrombus core suggests the further possibility that phagocytosis of RLF or its products has occurred. This is an attractive hypothesis, because it has been demonstrated that fibrin split products are chemotactic.

However, the autoradiographic demonstration of radioactivity within and on the surface of thrombi formed before and after RLF injection does not predict the success of thrombus detection by surface counting. A thrombus removed, sectioned and placed against photographic film is substantially different from a thrombus in a leg vein being sought by a surface counter. Thus, autoradiographs were consistently positive in thrombi removed as early as one hour after RLF injection, while surface counting techniques detected thrombi at substantially longer intervals after RLF injection. Clearly, surface detection requires that several conditions other than RLF incorporation be met.

**Figure 5**

Counts per second (CPS) detected by external counting in various locations at intervals after RLF injection. Thrombus was counted 2 cm below site of balloon occlusion; control, at same location over contralateral leg. Values shown are means and standard errors in 10 dogs. Thrombus had significantly greater (P < .05) activity than control at six hours and beyond.

**Figure 6**

Representative data in one dog. Surface counts at location 2 cm below site of occlusion (thrombus) and at 4 cm intervals to heel. Contralateral leg served as control. Thrombus/control difference was evident at six hours and became more marked thereafter.
Thrombus/Background Ratios

The thrombus/background ratio of radioactivity is a dynamic one because it depends upon both a numerator and a denominator which change with time. The ideal radiolabel would seek out thrombi, and attach to them, then rapidly clear from the body. Fibrinogen is not ideal in this regard, as it is cleared only after many hours. Therefore, the emergence of a favorable ratio depends upon the rate of incorporation of RLF into the thrombus and the speed of RLF clearance from the blood. Furthermore, if fibrinolysis is occurring with dispatch, the total radioactivity provided by the thrombus will fall with time.

Our data clearly reflect the fact that favorable thrombus/background ratios take time to emerge. Furthermore, and not surprisingly, they emerged earlier in thrombi formed in the presence of circulating RLF than in thrombi formed before RLF was injected. The well-counting data showed favorable ratios in the first category at three hours; in the second category, not until six hours in any thrombus and not until 12 hours in all thrombi.

Of course, as in the case of autoradiographs, well-counting ratios are not reliable predictors of the results to be obtained with surface counting. Background from large blood pools adjacent to a thrombus (or a bladder filled with radioactivity) may obscure a thrombus which has substantial radioactivity. But, in the case of the femoral vein of the dog, our surface counting data did compare reasonably well with the thrombus/blood information obtained by well counting. In all 10 instances, surface counting reflected thrombus/control difference at six hours, and beyond that time the differences became more marked. Since RLF was injected after thrombus formation, this is approximately the time course anticipated from the well-counting data. Why surface counting was, in fact, more favorable than anticipated must remain speculative beyond the observation that ligature-serum and balloon-serum thrombi may not behave in exactly the same manner.

These data, and the published experiences of others, indicate several limitations of the RLF method which should be recognized. The method is likely to be "insensitive" to thrombi located in regions with substantial blood flow (e.g. lower abdomen, lung, heart). Its sensitivity is enhanced by rendering the monitored area as bloodless as possible at the time of observation to decrease background counts; in the case of the lower extremities by elevating the legs as Kakkar has suggested.² ⁴ Trauma, with fibrinogen leakage into the tissues, also will obscure the presence of thrombi. This problem led us to abandon a technique for the production of thrombi which required surgical incision over the vein.

The precise technique used for surface counting is also of importance. Count rates can be substantially altered by variation in the geometry of probe application to a given site. The orientation of the leg and the site which is counted should be identical with each subsequent counting procedure.

Finally, there is legitimate question about what should serve as a criterion of venous thrombosis by this method. Specifically, what area should serve as "control," and what thrombus/control differences are significant? In man, opposite legs cannot be used with confidence since venous thrombosis is often bilateral. If the count rate measured at different sites along each leg is expressed as a percentage of the precordial count rate, then a difference of 15–20% between counting sites suggests the diagnosis of thromboembolism. But the reliability of these criteria requires further validation.⁶

Toxicity

Beyond the acceptable radiation exposure to the patient, the major hazard of the procedure is the potential transmission of hepatitis. This is indeed a major consideration for a material that may be applied to thousands of patients for screening. This hazard has been dealt with in England by obtaining fibrinogen from a small pool of donors who have donated blood for at least five previous transfusions without clinical or laboratory evidence of hepatitis occurring in the recipients in the succeeding six months. Each batch of fibrinogen is screened for Australian antigen. This same approach is being followed in this country. Therefore the risk of hepatitis should not pose a major barrier to RLF testing in humans.

Comments

Our investigations indicate that the RLF method has substantial promise as a means for detecting the presence of fresh venous thrombi in the lower extremities. They also have pointed out certain limitations of the method and raised other questions which need resolution before the technique can be applied in man with maximum effect. For example, we did not study thrombi which had been aged in vivo for hours to days;⁸ nor did we explore how heparin administration prior to RLF injection might influence the results.
This technique has potential for unravelling many of the mysteries surrounding venous thromboembolism. But widespread clinical use of the procedure should await establishment and dissemination of an adequate data base.

References
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