

COAGULATION OF SHEEP INTESTINAL AND PREFEMORAL LYMPH

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ABSTRACT

We have determined the most suitable method for the automated analysis of the clotting parameters in sheep intestinal and prefemoral lymph as defined by the Activated Partial Thromboplastin Times (APTT; measure of intrinsic coagulation pathway) and the Prothrombin Times (PT; measure of extrinsic coagulation pathway). As opposed to optical density systems, the use of a Fibro-System Fibrometer was found to provide the most consistent assessment of coagulation with the endpoint being the time to fibrin strand formation. We measured APTT in sheep intestinal and prefemoral lymph of 59.78 ± 7.69 seconds and 51.03 ± 10.49 seconds respectively. These values were more prolonged than those obtained from sheep blood plasma but only in the case of intestinal lymph were the differences significant ($p < 0.025$). Human blood APTT values were significantly less than both sheep blood ($p < 0.05$) and sheep intestinal ($p < 0.001$) and prefemoral lymph ($p < 0.01$). PT values were found to be 21.56 ± 1.14 seconds in intestinal and 22.00 ± 1.88 seconds in prefemoral lymph. These values were also significantly greater than those obtained from sheep blood (both $p < 0.001$). Human blood PTs were significantly less than both sheep blood ($p < 0.001$) and intestinal and prefemoral lymph (both $p < 0.001$). Measurement of APTT and PT values in intesti-

nal lymph and PT determinations in prefemoral lymph were not affected by storage in the refrigerator or freezer. There was some indication that APTT values in prefemoral samples were susceptible to storage artifacts; however, the differences in coagulation times were not significant.

It has been known for some time that lymph clots (1-8). This is to be expected since lymph is essentially an ultrafiltrate of blood plasma and as such contains all the protein factors present in blood. Clotting factors in lymph exist at concentrations approximately 10 to 90% of those in the vasculature depending on where in the body the lymph is collected from and the species involved (2,3,5,8-11). It follows then, that lymph clotting times are in general, longer than those for blood.

We and many others have used the lymphatic drainage model in sheep because this animal is large and permits relatively easy cannulation of various lymphatic vessels. Despite the popularity of the model, little information is available on the clotting properties of sheep lymph. In this paper, we report on studies that have assessed the coagulation profiles of sheep intestinal and prefemoral lymph. The Activated Partial Thromboplastin Time (APTT), an estimate of the intrinsic coagulation activity, and the Prothrombin Time (PT), an estimate of the extrinsic coagulation pathway, have

been measured and compared to values obtained with sheep and human blood plasma. In addition, we have determined the effects of different storage techniques on the lymph APTTs and PTs in order to test if lymph samples can be frozen or refrigerated prior to testing.

MATERIALS AND METHODS

Collection of lymph

Female sheep weighing between 23 to 27kg were used in these experiments. The sheep were fasted for 24 hours before surgery was performed. Anesthesia was induced with the intravenous administration of sodium pentobarbital (Somnotol, 20mg/kg, MTC Pharmaceuticals, Hamilton, Ontario) with additional smaller doses given as needed during surgery. All sheep were intubated (Portex 6.5-7.5) and allowed to breathe unassisted. For collection of intestinal lymph, the ileocolic mesentery was identified and exposed through a right flank transabdominal incision. The main mesenteric efferent lymphatic vessel originating from the terminal lymph node was cannulated 10 to 15cm downstream against the direction of lymph flow (Microbore Tygon tubing; O.D. 0.070in, I.D. 0.040in, Cole Parmer Instrument Co., Chicago). The catheter was secured in place by ties to the lymphatic vessel as well as to the surrounding tissue using silk sutures (4-0, Davis and Geck). For the collection of prefemoral lymph, the procedures outlined by Lascelles and Morris (12) were used. Polyethylene catheters (Clay Adams, PE-50 I.D. 0.023in, O.D. 0.038in) were inserted into the efferent prefemoral vessel approximately 4 to 6cm from the lymph node.

The catheters were externalized and the abdomen closed securely using #1 Dexon (Davis and Geck, Cyanamid, Montreal, Quebec). Lymph and blood samples were collected 2 to 6 hours after the surgery. By this time the sheep were conscious in that they could stand and respond to a noise stimulus. A few

sheep were allowed to recover for at least 24 hours before the collection of lymph or blood. The lymph was collected into plastic test tubes containing 3.8% sodium citrate (1 part anticoagulant/9 parts lymph) and was centrifuged at 1000 times g for 15 minutes to obtain the plasma. The samples were then separated into three aliquots. One aliquot was stored for one week in the refrigerator at 4°C. The second was stored for one week in the freezer at -20°C. The frozen samples were quick thawed at 37°C prior to testing. The third aliquot from the original sample was tested for APTT and PT immediately.

Collection of blood samples

Blood samples were collected by venipuncture under sterile conditions into 20cc syringes containing 3.8% sodium citrate (1 part anticoagulant/9 parts blood). Human blood was drawn from an arm vein, whereas sheep blood was obtained from the right or left jugular vein. The samples were centrifuged at 1000 times g for 15 minutes to obtain the plasma.

Analysis of coagulation

Clotting times were determined using an automated APTT test to measure the intrinsic pathway (Factors XII, XI, X, IX, VIII, V, II) and an automated Simplastin to measure the extrinsic pathway (Factors X, VII, V, II). General Diagnostics' automated APTT is a platelet factor 3 reagent (partial thromboplastin) with a particulate activator for the determination of activated partial thromboplastin times. General Diagnostics' automated Simplastin is a thromboplastin reagent for the measurements of prothrombin times. The reagents were used as directed in the manufacturer's instruction.

The clotting times were assessed with a Fibro-System fibrometer (Fibrometer Precision Coagulation Timer, Model #5, with automatic pipette and thermal preparation block, Division of Becton Dickinson and Co., Maryland,

USA). The endpoint of each pathway was the time to fibrin strand formation.

All reagents and samples were prewarmed to 37°C prior to use. Sample volumes of 0.1ml were placed into the fibrin cups. In the case of automated Simplastin, the sample was allowed to warm at 37°C for 3 min. The addition of 0.2ml Simplastin reagent by the automatic pipette started the timer and initiated the clotting cascade. Upon formation of a fibrin strand, the two electrodes submerged in the sample were bridged, thus completing the electrical circuit and stopping the timer. With respect to the APTT determinations, 0.1ml of sample and 0.1ml APTT reagent were incubated together for 5 min at 37°C in the fibrin cup to activate factors XI and XII. Following this, the addition of 0.1ml of 0.025M CaCl₂ triggered the timer and started the reaction. For APTT and PT determinations, each lymph or blood sample was measured 5 times and the mean of the 5 values calculated.

RESULTS

Comparisons of activated partial thromboplastin times in blood and lymph

The results of the APTT assays are illustrated in Fig. 1. Sheep blood plasma

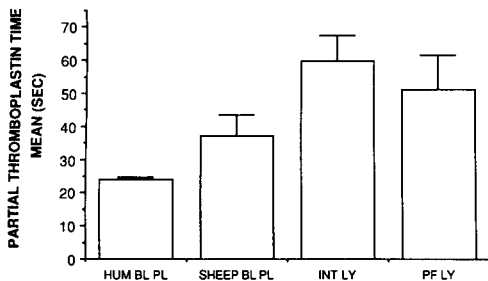


Fig. 1. Comparison of partial thromboplastin times in sheep and human blood and sheep lymph. HUM BL PL = human blood plasma; SHEEP BL PL = sheep blood plasma; INT LY = intestinal lymph; PF LY = prefemoral lymph. See Materials and Methods for details. Statistics have been assessed with the unpaired students *t*-test (see Results).

demonstrated an APTT of 37.26±6.08 sec. As expected, these were less than

those from intestinal (59.78±7.69 sec) or prefemoral lymph (51.03±10.49 sec); however, only in the case of intestinal lymph were these differences significant ($p < 0.025$). There was considerable variation in the clotting times of the prefemoral samples. In general, the variability in the clotting times for the lymph was greater than that of the blood determinations. There was no significant difference between the clotting times of intestinal and prefemoral lymph.

Human blood (collected from 6 individuals, 5 male, one female) demonstrated clotting times in the range of 23.88±0.62 sec) with much less variability than occurred in the sheep samples. These times were significantly less than both sheep blood ($p < 0.05$) and sheep intestinal ($p < 0.001$) and prefemoral lymph ($p < 0.01$).

Because of the possibility that there may have been some residual anesthetic effect in the sheep blood and lymph determinations, we also assessed APTT's and PT's in blood and lymph samples collected no earlier than 24 hours after the surgery. We could find no significant difference in any of the clotting parameters suggesting that an anesthetic effect was not a consideration in these studies (data not illustrated).

Comparisons of prothrombin times in blood and lymph

The results of the PT assays are illustrated in Fig. 2. Sheep blood plasma demonstrated a PT of 14.59±0.27 sec. These times were significantly shorter than those from intestinal (21.56±1.14 sec; $p < 0.001$) or prefemoral lymph (22.00±1.88 sec; $p < 0.001$). There was no significant difference between the clotting times of intestinal and prefemoral lymph. As in the case of APTT, the variability in the clotting times for the lymph was greater than that of the blood determinations.

The human blood demonstrated clotting times in the range of 12.02±0.33 sec). These times were significantly shorter than both sheep blood ($p < 0.001$)

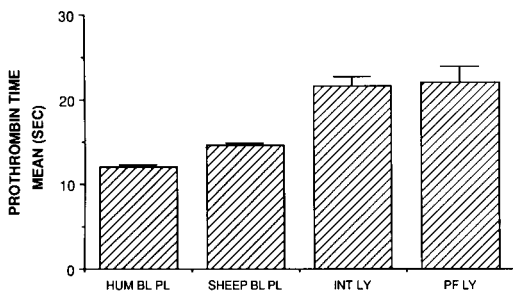


Fig. 2. Comparison of prothrombin times in sheep and human blood and sheep lymph. HUM BL PL = human blood plasma; SHEEP BL PL = sheep blood plasma; INT LY = intestinal lymph; PF LY = prefemoral lymph. See Materials and Methods for details. Statistics have been assessed with the unpaired students *t*-test (see Results).

and sheep intestinal ($p < 0.001$) and prefemoral lymph ($p < 0.001$).

As was the case with the APTT determinations, we could find no significant difference in any of the clotting parameters when comparing blood and lymph samples collected no earlier than 24 hours after the surgery with those collected between 2 and 6 hours after surgery (data not illustrated).

Effects of storage procedures on coagulation in blood and lymph

We also assessed whether blood and lymph samples from the sheep could be stored at 4°C in the refrigerator or could be frozen, and measured without alteration at a later date. The results are illustrated in Table 1. With intestinal lymph, there were no significant differences between the stored samples or the fresh collection when tested for APTT values. There was a tendency for prefemoral APTT values to be longer when stored (especially frozen); however, none of these differences was statistically significant with the relatively small sample size. In the case of PT assays, no storage induced differences were observed with either intestinal or prefemoral lymph. The same was true to APTT and PT measurements of sheep blood plasma, i.e., the storage of the samples had no effect on any of the clotting times.

However, there were significant differences between fresh, refrigerator and freezer stored human blood plasma samples with respect to both PT and APTT. Both refrigerator and freezer stored coagulation times were significantly more prolonged than those observed with fresh samples.

DISCUSSION

The coagulation of whole blood and lymph is a complex process with cellular and tissue elements interacting at several levels with the factor cascade. It is not clear what role cell products play in regulating lymph coagulation since some of the cells that play a pivotal role in the coagulation of blood, for example platelets, are largely absent from lymph. In any event, in the absence of cells, the action of the intrinsic and (or) extrinsic pathways result in clot formation in both blood and lymph samples. In blood plasma, the polymerization of fibrin results in a clot with a solid, gel-like consistency. In some cases, lymph plasma can produce this pattern but in many instances forms a very loose, watery mass with evidence of fibrin strand formation at the bottom of the test tube. This is similar to the clots that form in indwelling lymphatic catheters in sheep. In our experience, the fibrin strand can often be removed from the catheter with the resumption of lymph flow. It seems likely that the lack of a well defined clot may be due to the lower concentration of fibrinogen and other factors in the lymph plasma.

The nature of the lymph clot results in some difficulty in attempting to automate the APTT and PT test procedures. We originally used a spectrophotometric technique using a Coagulation Analyzer (Bio Data Corp, Willowgrove, PA) that stopped the timer following a change in optical density. This worked well with human and sheep blood samples but very often would not register any lymph coagulation despite fibrin deposition in the bottom of the tubes. For this reason, we went to a system in which

Table 1
The Effects of Storage on Activates Partial Thromboplastin Times (APTT) and Prothrombin Times (PT) of Sheep and Human Blood and Sheep Lymph

	BLOOD PLASMA		SHEEP LYMPH PLASMA	
	Human (n=6)	Sheep (n=9)	Intestinal (n=6)	Prefemoral (n=3)
Fresh APTT	23.88±0.62	37.26±6.08	59.78±7.69	54.37±16.60
Refrigerator APTT	29.53±0.59**	44.70±12.71	63.13±7.05	65.24±38.21
Freezer APTT	37.03±5.24*	36.66±6.18	53.33±6.46	82.13±34.52
Fresh PT	12.02±0.33	14.59±0.27	21.56±1.14	20.34±1.83
Refrigerator PT	14.61±0.44**	14.94±0.51	21.41±0.87	20.14±2.30
Freezer PT	17.54±1.10**	14.72±0.34	21.16±0.82	20.47±1.92

Comparisons were made between refrigerator or freezer stored samples and fresh collections (**p<0.001; *p<0.025).

the end point was a more direct measure of fibrin strand formation.

The Partial Thromboplastin Time measures the intrinsic coagulation activity. The test is based on the principle that, when a mixture of plasma and a phospholipid platelet substitute is recalcified, fibrin forms only if factors IX-XII, VIII, V, II, I are present. Using the Fibrometer, measurements of the intrinsic clotting pathway in lymph were possible although the clotting times were quite variable from animal to animal. The extra time required for the lymph clotting times compared to blood as measured by the APTT is most likely explained on the basis that the clotting factors in lymph are present at levels lower than those in the blood plasma (2,3,5,8-10). For a reason unknown to us, there was a tendency for the APTT values to be longer in intestinal lymph compared to prefemoral lymph; however, these differences were not statistically significant. The sheep blood APTT level of 37.26 was not very different from 31.9 sec observed by Gajewski and Povar (13).

The Prothrombin Time measures the extrinsic coagulation pathway. The PT values in lymph were significantly more prolonged than blood and there were no differences between intestinal and pre-

femoral lymph. As was the case with the APTTs, our value for sheep blood (14.59 sec) was similar to 14.75 sec recorded by Gajewski and Povar (13).

Human blood was in general more susceptible to storage artifacts than either sheep blood or intestinal lymph. In terms of PT determinations, storage of lymph samples in the refrigerator or freezer had no effect on coagulation. Storage of prefemoral lymph samples resulted in longer APTT values especially with freezing; however, these differences were not significant. It has been previously observed that the thromboplastic properties of the cellular material in lymph can be enhanced with freezing and thawing or increased contact with oxalate or citrate (2). Our samples, however, contained no cellular material. These data indicate that intestinal and prefemoral lymph samples can be stored in the refrigerator for at least 1 week prior to assay for PT values. For APTT investigations, frozen collections may have to be treated with more caution especially with prefemoral lymph.

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