Br. J. Pharmac. Chemother. (1966), **27**, 213–221.

OBSERVATIONS ON THE CONTENT OF KININOGEN, KALLIKREIN AND KININASE IN LYMPH FROM HIND LIMBS OF DOGS AND RABBITS

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(*Received February* 9, 1966)

Plasma kinins may play a role in inflammatory processes and possibly also in normal control of local blood flow and vascular permeability. Lymph will in many ways reflect the situation in the interstitial tissue spaces. Under normal conditions as well as during inflammation or tissue damage examination of locally collected lymph seems to be of interest in the evaluation of the possible local roles of kinins.

Hilton & Lewis (1955a, 1955b) found that the vasodilatation occurring in the activated submandibular salivary gland was related to the intact lymph drainage of the gland through which vasodilator material was drained away. Lymph contains most plasma constituents (Yoffey & Courtice, 1956). Schachter (1960) showed that thoracic duct lymph contained substrate for plasma kinin-forming enzymes and also kininase activity. Edery & Lewis (1962, 1963) reported that when lymph collected from dog hind limbs was incubated with pseudoglobulin, plasma kinins were formed and they interpreted this as being due to plasma kinin-forming activity in the lymph. Jacobsen & Waaler (1965) confirmed that plasma kinins were formed on incubation of dog limb lymph with pseudoglobulin, but they ascribed this to the presence of kinin-forming activity in the pseudoglobulin and of substrate for this enzymatic activity in the lymph. They were unable to demonstrate any kinin-forming activity in freshly taken samples of limb lymph.

In the present investigation lymph from hind limbs of anaesthetized rabbits and dogs has been examined for content of free kinins, of kininogen, of kinin-forming and kinin-destroying activity. Kininogen content has been examined in view of the presence in plasma of two substrates for kinin-forming enzymes (Jacobsen, 1966).

METHODS

Animals and anaesthetics. Albino rabbits of both sexes, weighing from 2.5 to 3.5 kg were used. They were anaesthetized by intravenous injections of 90 to 180 mg pentobarbitone sodium (Nembutal Natrium V.M., Abbott Laboratories Ltd., London) and 150 mg chloralose (α-chloralose puriss., E. Merck, Darmstadt, Germany). The anaesthesia was continued by giving 15–30 mg pentobarbitone sodium at intervals of from ½–2 hr. The concentrated solution of pentobarbitone was diluted 1:3 with a 0.9 per cent sodium chloride solution before injection. Chloralose was given as 1% solution in 0.4% sodium chloride solution.

Healthy male mongrel dogs weighing from 15 to 23 kg were used. They were premedicated with morphine (2 mg/kg) and anaesthetized with intravenously injected pentobarbitone, 30 mg/kg body weight being the initial dose. Small additional doses were given at intervals of from 1–2 hr. Artificial positive pressure ventilation was carried out in periods of the experiments.

Collection of lymph. One of the main femoral lymphatic vessels was cannulated in the inguinal region with a PE 10 intramedic polyethylene tubing (Clay-Adams Inc., New York, U.S.A.) with an internal diameter of 0.011″ and an outer diameter of 0.024″. The tubing was led out through the skin. The limb was exposed to gentle passive movements, and 0.45 or 0.9 ml. lymph drained by gravity into a graded siliconized glass tube containing 0.05 or 0.1 ml. 3.1% sodium citrate dihydrate solution respectively. Other visible lymphatic vessels in the region were tied off. The time needed for the lymph to pass through the piece of polyethylene collecting tube was of the order of from ½ to 1 min. During collection, and until the lymph was tested, the glass tube was kept in an ice-cooled water bath.

Plasma kinin-activity was usually evaluated on the rat uterus preparation or on the guinea-pig ileum preparation. Virgin rats weighing between 150 and 200 g were used for the uterus preparation which was suspended in a 5 ml. siliconized organ bath containing de Jalon solution at 29° C. The rats were injected intraperitoneally with 0.5 ml. 0.01% solution of diethyl-stilboesterol 18 hr before being killed. The uterus was usually stored for 24 hr at 4° C in de Jalon solution before being used for biological tests.

When the guinea-pig ileum was used, it was suspended in a 5 ml. organ bath containing Tyrode solution at 35° C.

Test samples were applied to the organ bath with a siliconized pipette at intervals of 4 min. The contact time of each sample was 60 sec. In this bath the uterus was usually sensitive to as little as 2–3 ng added bradykinin. Mepyramine maleate 10^{-4} g/l. (Anthisan®; May & Baker, Ltd., Dagenham) and methysergide 10^{-7} g/l. (Deseril®; Sandoz A.G., Basel, Switzerland) were added to the organ bath fluid for inhibition of histamine and 5-hydroxytryptamine respectively.

Kininase activity has been estimated by incubating 0.1 ml. lymph or plasma with 250 ng synthetic bradykinin in 0.35 ml. 0.1 M Tris-HCl buffer of *p*H 7.35 at 37° C. At intervals samples were taken out and tested on the rat uterus preparation and the time when all bradykinin activity had disappeared was noted.

Substrates for kinin-forming enzymes were estimated in plasma and lymph samples by the following procedures: (a) substrate for plasma kallikrein: 0.2 ml. plasma or lymph was incubated at 37° C with 0.1 ml. 2.7×10^{-2}M solution of disodium edetate and 0.1 ml. pseudoglobulin preparation from human plasma, which had been dialysed for more than 48 hr (Jacobsen, 1966). (b) Substrate for glandular kallikrein: 0.1 ml. plasma or lymph, 0.7 ml. 0.9% saline, 0.1 ml. 6.8×10^{-2}M solution of disodium edetate solution and 0.1 ml. undiluted cell-free human saliva were incubated at 37° C.

Aliquots were removed at intervals and tested on the rat uterus preparation. Maximal amount of kinin activity was reached after 1 min with plasma kallikrein (pseudoglobulin) and after 4 min with glandular kallikrein (saliva).

Plasma was usually obtained from citrated blood (one part 3.1% sodium citrate dihydrate to nine parts of blood). Sometimes heparinized blood was used. Blood was taken with silicone technique from a peripheral vein. Blood samples from the hind limb of the experimental animals were taken through a polyethylene cannula in the femoral vein. The blood samples were centrifuged at 4° C and 1,300 *g* for 30 min.

Pseudoglobulin preparation was prepared by ammonium sulphate precipitation of dog, rabbit or human citrated or heparinized plasma as described by Lewis (1958). The precipitate was suspended in distilled water and dialysed against running tap water for 60 hr (Jacobsen, 1966). The final volume of the solution of dialysed pseudoglobulin was about one-third of the original plasma volume. Pseudoglobulin preparation samples were stored at −20° C.

Stable plasma substrate preparation was prepared as described by Amundsen, Nustad & Waaler (1963).

Glass activation of plasma or lymph was carried out by agitating equal volumes of such samples and glass beads (ballotini) of diameter 0.1 mm for 3 min as described by Margolis (1958).

Protein in plasma and lymph samples was estimated using the de Biuret method (Gornall, Bardawill & David (1949).

Bradykinin. Synthetic bradykinin (BRS 640) (Sandoz, Basel, Switzerland) was used.

Glandular kallikrein. Human saliva without kininase-activity (Amundsen & Nustad, 1964) and Padutin® (Bayer, Leverkusen, Germany) were used as sources of glandular kallikreins.

Kininase inhibitor. Disodium edetate dihydrate (A. G. Fluka Buchs, Switzerland) in stock solutions of 2.7×10^{-2}M and of 6.8×10^{-3}M at *p*H 7.35 were used.

Erythrocytes in lymph samples were counted in a 1:10 dilution of the lymph in 0.9% saline.

Haemoglobin content in lymph samples was determined as cyanmethaemoglobin in a Zeiss spectrophotometer (Model PMQ II) at 540 mμ.

Paper electrophoresis was carried out according to Flynn & de Mayo (1951).

p*H measurements* were done with Astrup Micro Equipment (Type AME 1 c, Radiometer, Copenhagen).

Siliconizing of glassware and needles were carried out with Siliclad® (Clay-Adams Inc., New York). A 1% solution was used for glass and a 5% solution for metal, followed by drying at 100° C.

Dialysis was carried out with a Visking dialysis tubing 18/32 (Visking Dept., Union Carbide International Comp., New York) against tap water that had been passed through a glass wool filter

RESULTS

The flow in a lymph vessel from a limb is small and dependent upon movements of the limb. During anaesthesia with barbiturates the flow has been found to decrease (Polderman, McCarrell & Beecher, 1943). In our anaesthetized animals spontaneous flow from a limb lymph vessel was less than 0.1 ml./hr. Passive movements of the limb, however, caused a flow of some 1–2 ml./hr in dogs and of about 1–1½ ml./hr in rabbits. Lymph flow from a cannulated limb vessel also depends on the tissue temperature, decreasing with a fall in temperature and increasing with a rise (Courtice, 1946 ; Yoffey & Courtice, 1956). A constant body temperature of 38° C was maintained and local cooling of the limb was prevented.

Some characteristics of the lymph samples. It was necessary to measure and control the *p*H of the lymph as it might influence kinin formation and kinin inactivation. The *p*H of freshly collected lymph samples varied between 7.36 and 7.42. After storage of the samples at 4° C for some hr their *p*H increased to 7.8–7.9 and at −20° C to above 8.

Lymph collected from hind limbs of dogs and rabbits was found to have a protein content between 1.2 and 2.4 g/100 ml. On paper electrophoresis the various protein types of plasma appeared also in the limb lymph, although in somewhat different proportions, as would be expected (Perlmann, Glenn & Kaufman, 1943 ; Courtice & Morris, 1955). The albumin/globulin ratio was, for instance, higher in the limb lymph than in plasma.

The content of cells in the limb lymph samples varied, and was partially dependent on experimental procedures. In rabbit as well as in dog lymph about 3,000–5,000 erythrocytes/mm³ were usually found. Light passive movements of the limb did not cause any increase in this number. Free haemoglobin could usually not be detected in

centrifuged lymph samples from dog or rabbits. In our first experiments, however, it was observed that urethane, which is commonly used for anaesthesia in animals, produced a pronounced haemolysis in the animals' blood, and haemoglobin then occurred also in the lymph. Sodium pentobarbitone, injected in the undiluted form, and containing 0.09 g ethyl alcohol/ml., also produced some haemolysis. Erythrocytes contain kininases (Erdøs, Renfrew, Sloane & Wohler, 1963) and haemolysis will thus cause an increase in the kininase activity of plasma. Urethane was therefore avoided, and the Nembutal preparation diluted so as to prevent haemolysis.

The lymph samples from rabbits showed small tendency to clot, even when citrate was not present during collection (Jacobsen, Sele & Waaler, 1965). Dog lymph usually clotted when collected without citrate.

Free plasma kinins in lymph. Several authors have reported the presence of some free plasma kinins in plasma from normal individuals (Carretero, Nasjletti & Fasciolo, 1965; Oates, Melmon, Sjoerdsma, Gillespie & Mason, 1964). It is difficult to detect small amounts of plasma kinins in biological fluids, where kinins will usually be rapidly destroyed. Kinin-forming enzymes of the fluids may also easily be activated, i.e., on contact with a foreign surface during the collection procedure. With our collection procedure, using polyethylene tubing and siliconized, cooled glass tubes no free kinin could be detected in the lymph samples. Even when lymph volumes of up to 0.5 ml. were added directly to the isolated rat uterus in an organ bath of 5 ml., no contraction occurred. Lymph samples were also collected directly into 2 volumes of boiling ethyl alcohol, and the residue suspended in saline after evaporation (Hamberg & Rocha e Silva, 1957). Not even when this procedure was used could any free plasma kinin be detected in lymph samples from the rabbit and dog hind limbs.

Plasma kinin-forming activity in lymph. Edery & Lewis (1963) reported that lymph contains plasma kinin-forming activity. Their conclusion was based on the finding that kinin formation occurred on incubation of lymph with pseudoglobulin prepared from plasma. Pseudoglobulin has, however, recently been shown to contain only one of the two substrates for kinin-forming enzymes which are present in plasma, namely the one which reacts mainly with glandular kallikrein (Jacobsen, 1966). Furthermore, active plasma kallikrein might be present in pseudoglobulin preparations (Jacobsen, 1966). In tests for the presence of kinin-forming activity, dog or rabbit lymph was therefore incubated in siliconized glass tubes with various proportions of fresh human or dog plasmas that had been taken with silicone technique. Test samples of 0.1 ml. were taken out at intervals and added to the rat uterus preparation. No kinin formation was detected during such incubations, not even when disodium edetate was present in the mixture. Lymph samples were also incubated with a stable plasma substrate preparation (Amundsen, Nustad & Waaler, 1963) and with dog and human plasmas that had been heated to 56° C for 1 hr, without any kinin formation occurring. No kinin formation could be observed in undiluted or saline-diluted lymph samples which were kept at 37° C in siliconized glass tubes for 15 to 20 min, not even when disodium edetate was present as kininase inhibitor.

If dog lymph was agitated for 3 min with an equal volume of glass beads, however, a plasma kinin-forming activity could be detected on incubating 0.1 ml. agitated lymph

with 0.2 ml. dog plasma or dog lymph. A control mixture of 0.1 ml. agitated dog lymph and 0.2 ml. saline gave much less kinin development.

No kinin-forming activity could be detected in rabbit lymph or plasma on similar glass treatment, probably because plasma kallikrein is so rapidly inactivated in rabbit plasma (Jacobsen, 1966).

One must conclude from these findings that lymph contains no detectable active kinin-forming enzyme. Kinin-forming activity may develop, however, as in plasma, upon contact with glass.

Substrates for plasma kinin-forming enzymes in the lymph. Lymph samples from dog as well as from rabbit hind limbs developed kinnin activity when incubated with pseudoglobulin as a plasma kallikrein preparation or with reagents containing glandular kallikrein. Thus limb lymph apparently contains both those substrates which have been separated from plasma (Jacobsen, 1966). An evaluation of the amounts of the two substrates in dog and rabbit lymph was attempted by incubation with saliva and with pseudoglobulin from human plasma respectively. The results are shown in Table 1.

TABLE 1

CONTENT OF THE TWO SUBSTRATES FOR KININ FORMING ENZYMES AND OF PROTEIN IN PLASMA AND LYMPH FROM DOGS AND RABBITS

Content of substrates is given as amount of kinin (ng/ml.) that could be developed with excess of kinin forming enzymes (see Methods). The amounts of kinin formed are again given as the doses of synthetic bradykinin which had the same effect on the isolated rat uterus preparation. The values presented for substrate 2 are those obtained by subtracting the amount of kinin developed with pseudoglobulin from the larger amount of kinin developed with saliva

	Substrate 1			Substrate 2			Content of protein (g/100 ml.)		
Animal	in plasma	in lymph	content in lymph as % of content in plasma	in plasma	in lymph	content in lymph as % of content in plasma	in plasma	in lymph	content in lymph as % of content in plasma
Dog 1	800	10	(1·3)	1800	490	(27·2)	7·4	1·2	(16·2)
Dog 2	800	150	(18·8)	3200	2150	(67·2)	5·4	2·2	(40·7)
Dog 3	500	30	(6)	1700	770	(45·3)	6·0	2·0	(33·3)
Dog 4	500	100	(20)	2800	1800	(64·3)	5·2	1·8	(34·6)
Dog 5	600	40	(6·7)	1900	760	(40·0)	5·4	1·9	(35·2)
Rabbit 1	400	50	(12·5)	2100	800	(38·1)	6·4	2·2	(34·4)
Rabbit 2	350	30	(8·6)	1950	670	(34·4)	6·2	2·1	(33·9)
Rabbit 3	300	20	(6·7)	1200	300	(25·0)	6·0	2·4	(40)

Initially no kinin formation was detected on incubation of rabbit lymph with the pseudoglobulin preparation. However, if disodium edetate was present in the mixture, and if the first samples of the incubation mixture were tested after 15 sec and 60 sec respectively, kinin activity corresponding to about 20 to 50 ng bradykinin/ml. lymph could be detected. On further incubation, and especially when disodium edetate was not present, the kinin formed would rapidly disappear from the mixture. Presumably plasma kallikrein is very rapidly inactivated in rabbit lymph as it is in rabbit plasma (Jacobsen, 1966).

TABLE 2

KININASE ACTIVITY IN SIMULTANEOUSLY COLLECTED PLASMA AND LIMB LYMPH FROM VARIOUS RABBITS AND DOGS

The lymph samples collected as described in Methods. Blood for plasma preparation taken during lymph collection from femoral vein of the other hind limb. The kininase activity is expressed as inactivation time (min) of a standard amount of synthetic bradykinin (see text)

Animal	Inactivation time (min)	
	in plasma	in lymph
Dog 1	6	16
Dog 2	6	12
Dog 3	7	14
Dog 4	6	14
Dog 5	8	13
Rabbit 1	4	6
Rabbit 2	4	5
Rabbit 3	3	6

On incubating dog lymph with pseudoglobulin in siliconized glass vessels kinin development, corresponding to from 10 to 60 ng bradykinin/ml. lymph, occurred. When kininase activity was inhibited by adding 0.1 ml. 2.7×10^{-2}M disodium edetate solution/0.2 ml. lymph, kinin formation corresponding to as much as 150 ng/ml. lymph could be detected. Spontaneous kinin formation did not occur when lymph and this amount of disodium edetate were incubated alone.

Kininase activity of lymph. Kininase activity is present in thoracic duct lymph (Schachter, 1960) and in hind limb lymph (Edery & Lewis, 1963), but the activity is less pronounced than in plasma. In the present study kininase activity has been estimated as described in Methods. In samples of dog lymph the time taken for 0.1 ml. to inactivate 250 ng synthetic bradykinin (inactivation time) ranged from 12 to 16 min, in samples of dog plasma from 6 to 8 min (Table 2). In rabbit lymph the corresponding inactivation time was between 5 and 6 min and in plasma between 3 and 4 min. In experiments where haemolysis occurred a more rapid inactivation of bradykinin by the lymph samples was observed. Disodium edetate in a final concentration of 0.68×10^{-2}M gave a marked inhibition of the kininase activity in lymph. As much as 60 to 80% of the added bradykinin was then present after incubation for 30 min. The kininase inhibition by edetate was less marked in lymph samples with visible haemolysis.

DISCUSSION

In studies on lymph it may be important to pay attention to the *p*H of the samples since marked changes in their *p*H can occur on storage. Such changes may interfere with tests on kinin formation and inactivation.

The protein content of the lymph samples varied considerably. The relatively high protein content of some lymph samples may in part be due to the effect of barbiturate anaesthesia Polderman, McCarrell & Beecher, 1943), and partly to the effect of handling the limbs.

It was noted that rabbit lymph had a very small tendency to clot, and that it could be kept fluid for hours, even when no anticoagulant was present. This may be explained

by the finding of Jacobsen, Sele & Waaler (1965) that a blood coagulation-retarding component is present in rabbit lymph.

Free plasma kinins could not be detected in dog or in rabbit lymph. Even if some kinin formation did occur in lymph or in the interstitial fluid, one would not necessarily detect free kinins, as the time needed for collection of a lymph sample is quite long. Even relatively large amounts of kinin may be broken down before testing is possible or before kininase inhibitors can be added.

It has been shown by other workers that thoracic duct lymph contains substrate for plasma kinin forming enzymes (Schachter, 1960). In accordance with this we found such substrate to be present in dog as well as in rabbit lymph. The two different substrate fractions of plasma can both be detected in lymph, but their concentration there is lower than in plasma, in accordance with the lower content of proteins in lymph. That substrate which has the largest molecular weight (Jacobsen, 1966), has a relatively lower concentration in lymph than the other substrate, as would be expected if the two substrates in the lymph originate from plasma (Wasserman, Loeb & Mayerson, 1955).

Edery & Lewis (1963) concluded that lymph contains plasma kinin forming activity. Such activity was marked after injury of the limb, but even lymph from an intact limb contained some activity. If lymph itself should contain both forming enzyme and substrate, one would expect kinin formation to occur in lymph without addition of another substrate preparation. Incubation of lymph alone or incubation of lymph diluted with saline did not result in any kinin formation. Even in the presence of disodium edetate as kininase inhibitor such formation could not be detected. Nor did lymph induce any kinin formation in fresh plasma. Kinin forming activity was thus not detected in the present investigation. The kinin formation occurring on incubation of lymph and pseudoglobulin is most likely a result of the reaction between plasma kallikrein in the pseudoglobulin preparation and the corresponding substrate in the lymph (Jacobsen, 1966).

In rabbit plasma and lymph the amount of substrate for plasma kallikrein is difficult to evaluate because of the high plasma kallikrein inhibitory effect and the high kininase activity. Short incubation periods, excess of plasma kallikrein and the presence of disodium edetate as kininase inhibitor will reveal the presence, but perhaps not the total content, of such substrate in rabbit plasma and lymph.

It appears as if all the plasma components related to kinin formation and inactivation, namely the two kininogens, inactive kallikrein and one or more kininases, are found in lymph. The lymph concentration of these plasma components are lower than in plasma, as it is for other proteins. It is reasonable to believe that these protein components in lymph are derived from plasma.

SUMMARY

1. No plasma kinin activity and no kinin-forming activity could be detected in lymph collected from the hind limbs of anaesthetized dogs and rabbits.

2. The lymph samples from both animal species were, however, found to contain the two different substrates for kinin-forming enzymes, which have previously been shown to be present in plasma.

3. The concentrations of the substrates were lower in lymph than in plasma. The substrate with the larger molecular weight was present in lymph in the lower concentration relative to that in plasma.

4. Kininase activity of the lymph samples was less pronounced than the corresponding activity in plasma.

This work has been carried out during a period when the author has been a Research Fellow of the Norwegian Research Council for Science and the Humanities. Financial support from this Council, from Eli Lilly and Company, Indianapolis, U.S.A., from the Nansen Foundation and from the Norwegian Council on Cardiovascular Diseases is gratefully acknowledged. I also wish to thank M. Kriz for skilful technical assistance.

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