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Red Blood Cell Catabolism in Lymph Nodes of the Sheep and Rat: Quantitation

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Summary

Estimates have been made of the number of red blood cells catabolised in the lymph nodes of the sheep and the rat. These estimates were based on observations on the output of bilirubin in intestinal lymph of the sheep and on bilirubin output in thoracic duct lymph of the rat.

It was calculated that some 2 to 15 x 10^9 red blood cells were catabolised in a 24 hour period by the lymph nodes of the intestinal region of the sheep, representing approximately 3% of all the red cells destroyed in this period. Similarly, 0.9 to 4.6 x 10^8 red blood cells were estimated to be catabolised every 24 hours in the lymph nodes of those areas drained by the thoracic duct of the rat, representing approximately 6% of all the red cells destroyed in a 24 hour period.

Calculations extending these results to include all areas of the body in both species indicated that the lymph nodes may account for some 6–7% of the total number of red blood cells catabolised in the animal.

Introduction

Red blood cells are phagocytosed by cells of the reticuloendothelial system (reticular cells, histiocytes and monocytes) as well as by polymorphonuclear leukocytes. However, the actual sites of catabolism of senescent red cells are at present uncertain. Studies using labelled red blood cells suggest that experimentally damaged cells are largely removed from the circulation by the liver and spleen in both animals and man (1, 2, 3, 4, 5), while physiologically-aged red blood cells may be removed by the bone marrow (6, 7, 8, 9). Recent findings from our laboratory suggest that the lymph node may also be an important site of catabolism of senescent red blood cells (10).

In the intact animal red cell haemoglobin, either endogenously formed or exogenously administered, is converted almost quantitatively to bilirubin (11). From the determination of the rate of bilirubin production in man and animals, it is therefore possible to calculate the number of red blood cells being catabolised per day, and the mean red cell life span, if the total number of red cells in the body is known. Estimates of this nature have been made in man by *Berk* et al. (12). These authors found close agreement between the mean red cell life span determined from estimates of the rate of bilirubin production per day and values obtained with the use of ^{51}Cr-labelled red blood cells.

In the present study an attempt has been made to quantify the number of red blood cells catabolised in lymph nodes each day from observations on the bilirubin output in post-nodal lymph of the sheep and rat.

Materials and Methods

Animals

Non pregnant Merino and Merino cross-bred ewes or wethers, aged between two and four years, were used in the experiments. The animals were housed indoors in individual metabolism cages and fed a mixture of lucerne hay and oats. Water was provided *ad libitum.*

Surgical Procedures

Various lymphatic ducts in 25 sheep were cannulated according to the methods referred to in *Smith, McIntosh* and *Morris* (13). The number of sheep and the lymphatic preparations established

in each were as follows: An efferent lymphatic duct of the popliteal lymph node was cannulated in each of 13 sheep. Efferent and afferent lymph of the hepatic lymph node were obtained from three sheep by cannulating one or two of the afferent lymphatic ducts from the node in each sheep (in one of these sheep the efferent lymphatic duct ceased flowing the day after surgery and values for this duct are not included in the paper). A similar procedure was followed to obtain efferent and afferent lymph of the prescapular lymph node in one sheep. In an additional four sheep, three efferent and one afferent lymphatic ducts of the prescapular node were cannulated. Lymph was also collected from several afferent lymphatic ducts to the prefemoral lymph node in two sheep and from the efferent lymphatic duct of the inguinal node in each of two sheep. An indwelling cannula was placed in an external jugular vein of each sheep at the time of the lymphatic cannulations.

In addition to the above 25 sheep, intestinal lymph was collected from 7 sheep following the procedure of *Lascelles* and *Morris* (14). The intestinal lymph was recirculated in each sheep through an indwelling jugular cannula.

The sheep were allowed a minimum of two to three days to recover from the effects of anaesthesia and surgery before regular sampling of blood and lymph was undertaken. Lymph samples were collected into graduated measuring cylinders containing 0.01 ml of a 1000 i.u./ml heparin solution (Pularin, Evans Medical Australia, Ltd.). Lymph was collected in this manner at regular intervals for periods of 30 to 60 minutes and lymph flow estimated from the volume of lymph collected. Blood samples were taken from the jugular cannula in the middle of each period of lymph collection.

The thoracic ducts of four Wistar strain (random bred, closed colony) rats were cannulated by the method of *Bollman, Cain and Grindlay* (15) and samples of thoracic duct lymph collected for analysis over the following 1–5 days. Blood samples were obtained from an indwelling femoral vein cannula in each rat.

Biochemical Analyses

(a) *Total Bilirubin* – Total bilirubin in lymph and blood plasma was determined using a modification of the *Jendrassik* and *Grofts* alkaline diazo-coupling method as described by *Broderson* and *Jacobsen* (16). The optical density readings (0D600) obtained using this method were in the range of 0.010 to 0.030 for plasma (mean coefficient of variation for 20 observations, 6.9%), 0.020 to 0.150 for efferent lymph (mean coefficient of variation for 20 observations, 4.0%), and 0.000 to 0.015 for afferent lymph (mean coefficient of variation for 20 observations, 19.7%). A bilirubin standard was prepared from pure bilirubin (Sigma Chemical Co., USA) according to the methods previously described (17, 18).

(b) *Albumin Determination* – The single radial immunodiffusion technique (19) was used to determine the concentration of albumin in lymph and blood samples. Monospecific anti-ovine albumin and pure ovine albumin were prepared as described previously (10).

Histochemistry

Prefemoral, prescapular, renal and hepatic lymph nodes were removed from two normal sheep. The nodes were fixed in formol-saline, embedded in paraffin and 4 μm sections stained for ferric iron (20) and bilirubin (21). A serial section was also stained with haematoxylin and eosin to determine structure and cell differentiation.

Quantitation of the Number of Red Blood Cells Catabolised

Average values for haemoglobin and red cell concentration in whole blood, total blood volume, mean red cell life span and the estimated number of red blood cells catabolised per day in the sheep and rat are shown in Table 1. Using these values, it was possible to determine the number of

red blood cells required to be catabolised to produce the output of bilirubin observed in post-nodal lymph of the sheep and rat.

Each molecule of haemoglobin (molecular weight = 64,485) contains 4 haeme groups and therefore gives rise to 4 molecules of bilirubin (molecular weight = 585). This means that for every gram of red cell haemoglobin catabolised, 36.2 mg of bilirubin is produced. Thus, for every 12×10^9 red cells catabolised in the sheep, 0.12 g of haemoglobin is degraded and 4.34 mg of bilirubin is produced. It follows that for every milligram of bilirubin produced, 0.028 g of haemoglobin is degraded and this would require the catabolism of 2.77×10^9 red blood cells. Similarly in the rat, for every 9×10^9 red cells catabolised, 0.14 g of haemoglobin is degraded and 5.07 mg of bilirubin is produced. Again, for every milligram of bilirubin produced, 0.028 g of haemoglobin is degraded and this would require the catabolism of 1.77×10^9 red cells in the rat.

Table 1 Average values for blood volume, red blood cell (RBC) concentration and haemoglobin concentration in whole blood of the sheep and rat. The mean life span for red blood cells for both species is also presented. Values taken from *Schalm* (22).

Species	Sheep	Rat
Blood volume (ml)	2260*	24†
Haemoglobin concentration (g/100 ml)	12	14
RBC concentration ($\times 10^9$/ml)	12	9
Mean RBC life span (days)	100	65
Number of RBC catabolised ($\times 10^{10}$/day)	27	0.33

**Quin*, unpublished data; † *Altman* and *Dittmer* (23)

The bilirubin output in lymph was determined by multiplying the concentration of bilirubin in lymph by the lymph flow per day. The number of red blood cells required to be catabolised to produce this bilirubin was determined by multiplying the bilirubin output (mg) in sheep by 2.77×10^9 (red cells catabolised per mg of bilirubin produced), and in rats by 1.77×10^9 (red cells catabolised per mg of bilirubin produced).

Results

Total Bilirubin Concentrations in Lymph

The total bilirubin concentrations observed in efferent and afferent lymph of the prescapular and hepatic lymph nodes, efferent lymph from the inguinal lymph node and afferent lymph to the prefemoral are shown in Table 2. In accord with previous results (10), the total bilirubin concentrations in efferent lymph of the hepatic, inguinal and prescapular lymph nodes were higher than those observed in the corresponding blood plasma samples while the total bilirubin concentrations in afferent lymph from three regions were normally less than those of blood plasma. It is unlikely that the bilirubin was derived from the capillary and venular filtrate within the node since the bilirubin lymph:plasma concentration ratio was considerably higher than that observed for its carrier protein albumin.

Table 2 The total bilirubin concentrations observed in various sources of lymph and in blood plasma of the sheep, together with the lymph:plasma concentration (C_L:Cp) ratios for bilirubin and albumin. Values shown are means ± standard errors with the number of animals in each group shown in brackets.

	Concentration (μg/ml)	C_L:Cp Ratio Bilirubin	C_L:Cp Ratio Albumin
Plasma (11)	1.20 ± 0.12		
Afferent prescapular (2)	0.75 ± 0.05	0.82 ± 0.32	0.58 ± 0.02
Efferent prescapular (4)	2.50 ± 0.33	2.57 ± 0.87 (2)	0.64 ± 0.02
Afferent hepatic (3)	0.60 ± 0.00	0.54 ± 0.03	0.99 ± 0.08
Efferent hepatic (2)	1.28 ± 0.43	1.21 ± 0.42	0.97 ± 0.01
Afferent prefemoral (2)	1.09 ± 0.09	0.64 ± 0.08	0.51 ± 0.00
Efferent inguinal (2)	3.86 ± 1.06	3.50 ± 0.50	0.66 ± 0.03

Histochemistry

The results of histological examination of the prescapular, prefemoral, renal and hepatic lymph nodes were similar to those reported for the mesenteric and popliteal lymph nodes (10). Reticuloendothelial cells, principally within or close to the lymphatic sinuses, were found to contain granules in their cytoplasm which stained for free iron and bilirubin. There was less evidence for red blood cell catabolism in the renal and hepatic lymph nodes, despite the presence of large numbers of red cells in the sinus areas of the renal node. In this connection, the concentrations of total bilirubin in efferent lymph from the renal and hepatic lymph nodes were lower than the comparable concentrations in efferent lymph from the prescapular and inguinal nodes (see Table 2) and from the popliteal and intestinal nodes (cf. 10). This finding would indicate that the renal and hepatic lymph nodes catabolise fewer numbers of red blood cells than lymph nodes in other regions of the sheep.

Quantitation of the Number of Red Cells Catabolised in Lymph Nodes

The bilirubin output and the number of red blood cells required to be catabolised to produce this output in efferent intestinal and popliteal lymph of the sheep and in the thoracic duct of the rat are given in Table 3. It can be seen that sizeable numbers of red blood cells would need to be catabolised to produce the amounts of bilirubin observed in these fluid pools. While there was considerable variation between animals, it was calculated that an average of 6.45×10^9 red cells per day would be catabolised in the lymph nodes of the intestinal region of the sheep and 0.20×10^9 red cells per day would be catabolised in the lymph nodes supplying the thoracic duct lymph in the rat. If it is assumed that the average bilirubin concentration in all post nodal lymph in the sheep is similar to the observed concentration of 2.00 μg per ml in intestinal lymph (the average bilirubin concentration in efferent lymph from seven regions in a total of 33 sheep was 2.90 μg/ml), then the total bilirubin output in lymph would be 7.0 mg per day (3,500 ml lymph per day x 0.20 mg bilirubin per 100 ml of lymph). The figure for total lymph flow used in this calculation was derived from previous observations on similar sheep kept under the same conditions in these laboratories (cf. *Yoffey and Courtice*, 24). This output of bilirubin (7.0 mg) would require the catabolism of 19.4×10^9 red cells, or an estimated 7% of the total red cells catabolised per day in the sheep. A similar calculation in the rat, assuming all the lymph in the rat to have a similar bilirubin concentration to that observed in thoracic duct lymph (3.40 μg/ml) yields an output of 0.08 mg per day (24 ml lymph per day x 0.34 mg bilirubin per 100 ml of lymph). The figure for total lymph flow in the rat was estimated from the thoracic duct lymph flow (4 rats, mean flow = 1.33 ± 0.33 ml/-hr). This output of bilirubin would require the catabolism of 0.14×10^9 red cells per day, or an estimated 6% of the total red blood cells catabolised each day in the rat.

Table 3 The total bilirubin in efferent lymph from the intestinal region and from the popliteal lymph node in the sheep, and in thoracic duct lymph of the rat. Computed values for the amount of haemoglobin, the number of red blood cells (RBC), and the precentage of the total RBC catabolised in the animal each day required to produce this amount of bilirubin are also shown. Values shown are means ± standard errors with the range of values shown in brackets.

	Bilirubin Output in lymph (mg/day)	Haemoglobin degraded (mg/day)	RBC catabolised ($\times 10^9$/day)	% Total RBC catabolised (per day)
Sheep				
Efferent intestinal	2.33 ± 0.58	66 ± 17	6.45 ± 1.61	2.4 ± 0.6
(7)	(0.8 - 5.3)	(20 - 150)	(2.1 - 14.6)	(1 - 5)
Efferent popliteal	0.39 ± 0.05	11 ± 2	1.08 ± 0.16	0.5 ± 0.1
(13)	(0.2 - 0.7)	(5 - 24)	(0.47 - 2.38)	(0.2 - 0.7)
Rat				
Thoracic duct	0.11 ± 0.05	3 ± 1	0.20 ± 0.09	6.0 ± 3.0
(4)	(0.02 - 0.26)	(1 -7)	(0.09 - 0.46)	(1 - 14)

Discussion

In the human and most other mammalian species, between 0.8 and 1.0% of the total red blood cells in the body are destroyed every 24 hours. In this study, it was estimated that some 6–7% of these red blood cells are catabolised in lymph nodes. Notwithstanding, several assumptions were made in this quantitation. It was assumed that all the bilirubin formed within the lymph node reached the blood stream via the efferent lymph, that the bilirubin present in efferent lymph was entirely derived from catabolism of red blood cells within the lymph node, and that surgical interserence during the cannulation of the lymphatic duct did not alter the rate of catabolism in the lymph node. The first assumption may be considered to be essentially correct since the bilirubin in efferent lymph was shown to be bound to serum albumin (10) and *Courtice* and his co-workers (25, 26) have shown that all the labelled albumin introduced into the connective tissue spaces is returned to the blood stream by way of the lymphatic system. However, not all the bilirubin in efferent lymph can be assumed to be derived from red blood cell catabolism in lymph nodes. It is evident that some of the bilirubin would be derived from both the afferent lymph and the capillary and venular filtrate within the lymph node. The low concentrations of bilirubin in blood plasma and in afferent lymph would nevertheless suggest that only relatively small amounts of bilirubin would be derived from these sources. Although surgery would have had some influence on normal physiology, it would be unlikely to have much influence on the output of bilirubin in the intestinal lymph of the sheep and in thoracic duct lymph of the rat since the sites of cannulation were well removed from the sites of lymph formation. In addition, the low concentrations of bilirubin observed in afferent lymph to the various lymph nodes would suggest that there was little, if any, post-operative haematoma formation. The presence of catabolic products in the lymph nodes from normal sheep would also remove the suspicion that red blood cell catabolism in lymph nodes was a surgically-induced phenomenon.

In recent years it has been shown that the activity of haeme oxygenase, the microsomal enzyme complex which converts haeme to bilirubin IX (27), can be stimulated in tissue macrophages by the presence of haeme compounds (28). This finding would suggest that the conversion of haeme to bilirubin could occur in any macrophage which engulfed and lysed red blood cells. The sites of catabolism of red blood cells would then be determined by the presence of cells in the tissue or organ with the ability to recognize and phagocytose senescent red blood cells rather than the presence of macrophages with the ability to convert haeme to bilirubin. This raises the question of what determines the recognition and phagocytosis of sensecent red blood cells. *Bunn* (29) suggested that the most important single determinant in red blood cell survival was deformability; this is the ability of red cells to undergo changes of shape enabling them to traverse the narrow passages in the microcirculation. *Bessis* (30) added that adherence of aged red cells to the surface of macrophages may also play a considerable role since normal red blood cells show no sign of adherence to phagocytes.

The structure of the lymph node, with its arrangement of trabeculae, reticulum and macrophages, designed primarily to function as a filter, would provide the animal with an excellent system of detecting red blood cells which had lost their deformability and consequently adhered to the macrophages. Indeed, the fact that less than 10% of the total catabolism of red blood cells occurs in lymph nodes is more probably due to the low numbers of red blood cells exposed to trapping within the nodes rather than a function of the catabolic ability of the macrophages within the nodes. The increase in bilirubin concentration in efferent lymph from the popliteal lymph node observed following the injection of isologous red blood cells by way of the afferent lymph (10) would lend support to this hypothesis.

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