Acta vet. scand. 1964, *5*, 370—383.

From the Medical Department for Non-Ruminants, Royal Veterinary College and the Division of Radiobiology, Research Institute of National Defence, Stockholm.

MEGAKARYOCYTES, THROMBOCYTES, AND BLOOD-CLOTTING TIME IN DOGS WITH EXPERIMENTAL HEPATITIS CONTAGIOSA CANIS*)

By

Gert Lindblad and *Anders W. Bäckgren*

In the extensive literature on Hepatitis contagiosa canis (H.c.c.) (Rubarth's disease) most authors, in describing the clinical picture and autopsy findings, mention an increased tendency to bleed. Petechiae in the gingiva and the submucosa of the mouth, bleeding into the intestine and stomach, loss of blood into the abdomen, and subcutaneous haemorrhages are among the disorders described (*Rubarth* 1947, *Smith* 1951, *Gillespie et al.* 1964, and others).

A prolonged bleeding-time in dogs with H.c.c. has been demonstrated by *Poppensiek* (1952). The cause of this is presumed to be the interference of the liver damage with the synthesis of prothrombin and fibrinogen, and concurrent release of heparin from the injured liver. *Coffin & Cabasso* (1953) have observed a prolonged clotting time in dogs with H.c.c.

In the description of the histopathological findings in fatal cases, *Rubarth* (1947) reports a marked decrease in the number of megakaryocytes in the bone-marrow.

We report here a study of clotting-time, prothrombin index, and total number of thrombocytes per mm^3, and quantitative and qualitative determinations of megakaryocytes in dogs with experimentally produced H.c.c.

---

*) Supported by grants from "Anslag för främjande av medicinsk forskning vid Veterinärhögskolan".

As quantitative determination of megakaryocytes in bone-marrow smears gives very uncertain results (*Berman et al.* 1948, *Craddock et al.* 1955, and personal observations), the megakaryocytes were studied in sections of biopsy specimens from ribs.

MATERIAL AND METHODS

17 susceptible dogs of mixed breed, males and females, whose ages ranged from 3 to 6 months were used in the experiments. The virus material was an H.c.c. strain*) (titre 7.5 $TCID_{50}$/ml); the inoculation was made by the intraperitoneal route, 4 ml of undiluted virus culture being injected. The severity of the symptoms produced was assessed by repeated determinations of total leucocyte counts, body-temperatures, and glutamic-oxaloacetic transaminase (G.O.T.) and ornithine carbamyl transferase (O.C.T.) levels.

The clotting-time was determined by the method described by *Best & Taylor* (1945) and the examination was made at about +20°C. The prothrombin-time was determined by the method of *Quick-Lehman* (1941) and the thrombocytes were counted by *Thorell's* (1962) technique.

The megakaryocytes were studied in rib-marrow sections, and the assessment was made without any knowledge of the origin of the preparation.

Specimens of ribs were resected under anaesthesia induced with thiopentone sodium. An incision was made through the skin, fat, and muscles, the periost was dissected free, and a piece of rib, about 4 cm in length, was cut off with a bone forceps. The cut piece was immediately fixed in Zenker's solution and formalin. The resection was made once only from each rib and the same rib was chosen in all the dogs. The operation did not affect the dogs to any noteworthy degree. No painful breathing or pressure over the chest were noted. Only minor reactions in the operation area were seen at autopsy, and within a few days of operation the defect in the rib was filled with new tissue and showed relatively good stability.

Resections of ribs were made 8 times in dogs nos. 1—5 and 7 times in dogs nos. 15—17. To ascertain the effect of the operation on the megakaryocytes, these were studied in 2 non-inoculated dogs which were operated upon in parallel with dogs 1—5.

The megakaryocytes were examined in sections 3 μ in thickness, stained with azure eosinate (*Lillie* 1948). The number of megakaryocytes was counted in rib-marrow sections at magnification 300 times. The examined section surface was 1.6 cm^2 per dog and day of sampling.

---

*) Obtained from *R. Salenstedt,* V.M.D., National Bacteriological Laboratory, Stockholm.

RESULTS

The clinical picture was fairly uniform in all the dogs, as regards such symptoms as anorexia and deterioration of general condition. The results referring to total leucocyte counts, G.O.T., and O.C.T. are summarized in table 1.

Table 1. Total leucocyte counts, G.O.T. and O.C.T. levels in dogs with experimentally produced H.c.c. Figures in parentheses refer to day after inoculation.

Dog no.	G.O.T. Maximum value	O.C.T. Maximum value	Total leucocyte Minimum value
1	40 (4)	6.1 (4)	3700 (4)
2	101 (2)	50.5 (2)	4200 (4)
3	134 (6)	40.2 (6)	2900 (4)
4 died (5)	510 (4)	98.5 (4)	2800 (4)
5	53.5 (6)	13.9 (4)	6900 (4)
6	28 (2)	4.4 (2)	7100 (2)
7	42.5 (2)	2.8 (4)	10.400 (2)
8 died (4)	98.5 (3)	3.9 (3)	3300 (3)
9	670 (5)	142 (7)	2200 (5)
10	535 (5)	78.4 (7)	2000 (5)
11 died (4)	76.5 (3)	15.8 (3)	6200 (3)
12 died (4)	98.5 (3)	29.2 (3)	6100 (3)
13	83 (6)	19.5 (4)	1800 (3)
14 died (4)	225 (4)	59.5 (4)	2400 (3)
15	37 (12)	5.4 (5)	4200 (5)
16	170 (7)	98 (7)	5300 (5)
17 died (9)	400 (7)	196 (7)	1900 (5)

The results for clotting-times and thrombocyte counts are shown in figs. 1 and 2. Dogs nos. 8—14 were reared without colostrum. Four of these 7 dogs died within 4 to 5 days of the inoculation. The three survivors, nos. 9, 10, and 13, had the longest clotting-times (37, 20, and 24 minutes, respectively; in dogs nos. 9 and 10 on the 5th and in dog no. 13 on the 6th day after inoculation.

Before the inoculation, no dog had a clotting-time exceeding 8 minutes.

The lowest thrombocyte counts were noted on days 4—7 after the inoculation (fig. 2).

For normal prothrombin-time in the formula:

$$\text{prothrombin index} = \frac{\text{normal prothrombin-time} \times 100}{\text{found prothrombin-time}},$$ was

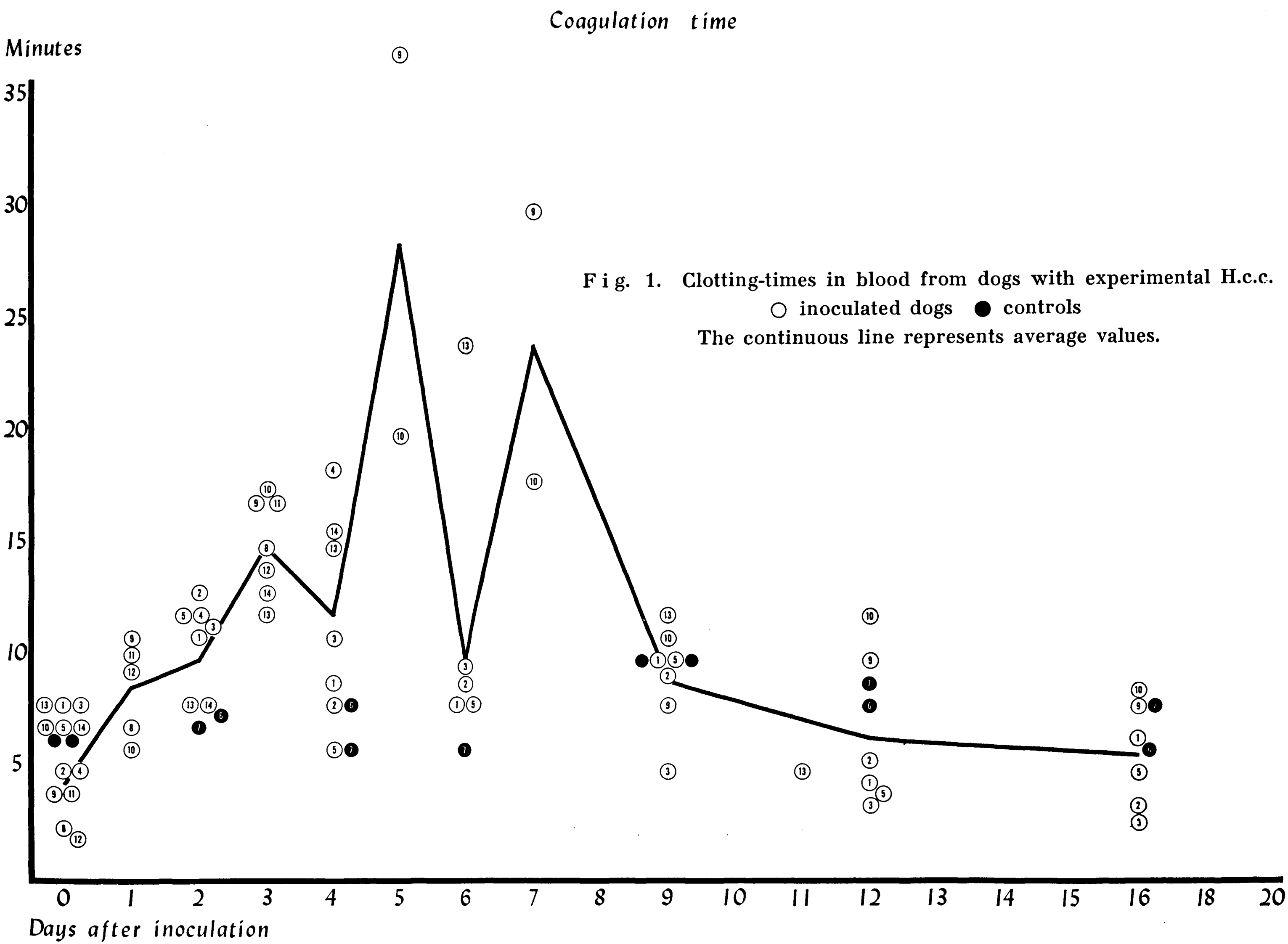


Fig. 1. Clotting-times in blood from dogs with experimental H.c.c.
○ inoculated dogs ● controls
The continuous line represents average values.

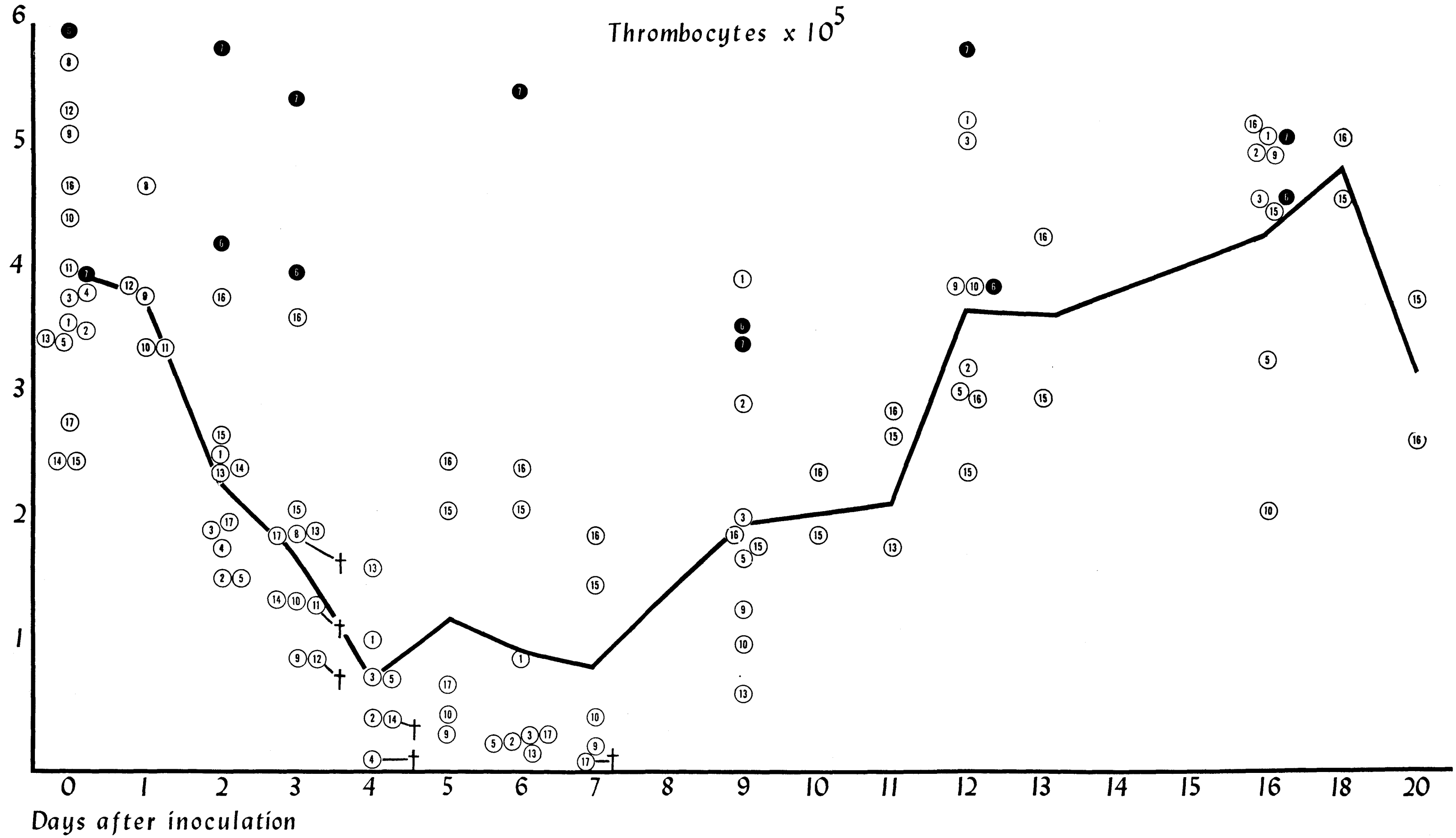


Fig. 2. Number of thrombocytes per mm³ of blood from dogs with experimental H.c.c. ○ inoculated dogs ● controls
The continuous line represents average values.

used the prothrombin-time that the respective dog hade before the inoculation. Some lowering of the prothrombin index occurs a few days after the inoculation. The lowest value, 62, was found in dog no. 10 on the 7th post-inoculation day. The minimum values for prothrombin index are set out in table 2.

Table 2. Minimum values for prothrombin index in 12 dogs with experimentally produced H.c.c. Normal value = preinoculation value = 100. The figures in parentheses refer to number of days after inoculation.

Dog no.	Prothrombin index		Dog no.	Prothrombin index
1	88 (4, 7)		8	81 (3)
2	82 (4, 6)		9	78 (7)
3	82 (4)		10	62 (7)
4	78 (4)		11	88 (3)
5	83 (4)		12	88 (3)
6	89	control	13	77 (11)
7	94—114	control	14	100

The results of the quantitative studies of the megakaryocytes are shown in fig. 3.

Qualitative estimation of the megakaryocytes

Before inoculation: All developmental stages of megakaryocytes were represented. The predominating cell type consisted of large megakaryocytes with usually markedly lobated nuclei and very wide thrombocyte-producing cytoplasmic zones. There were also degenerating cells with fairly homogeneous eosinophilic cytoplasm and as a rule pycnotic nuclei. In some degenerating cells the cytoplasm was reduced to a very narrow zone (fig. 4).

2 days after inoculation: There seemed to be a relative increase in the number of degenerating cells. These were, almost without exceptions, large cells with comparatively large pycnotic nuclei and wide cytoplasmic zones, which in many cases were eosinophilic.

4—5 days after inoculation: Very marked degenerative changes were present. There were only an insignificant number of thrombocyte-producing megakaryocytes.

6—7 days after inoculation: The degenerative changes predominated. At the same time there was some increase in the number

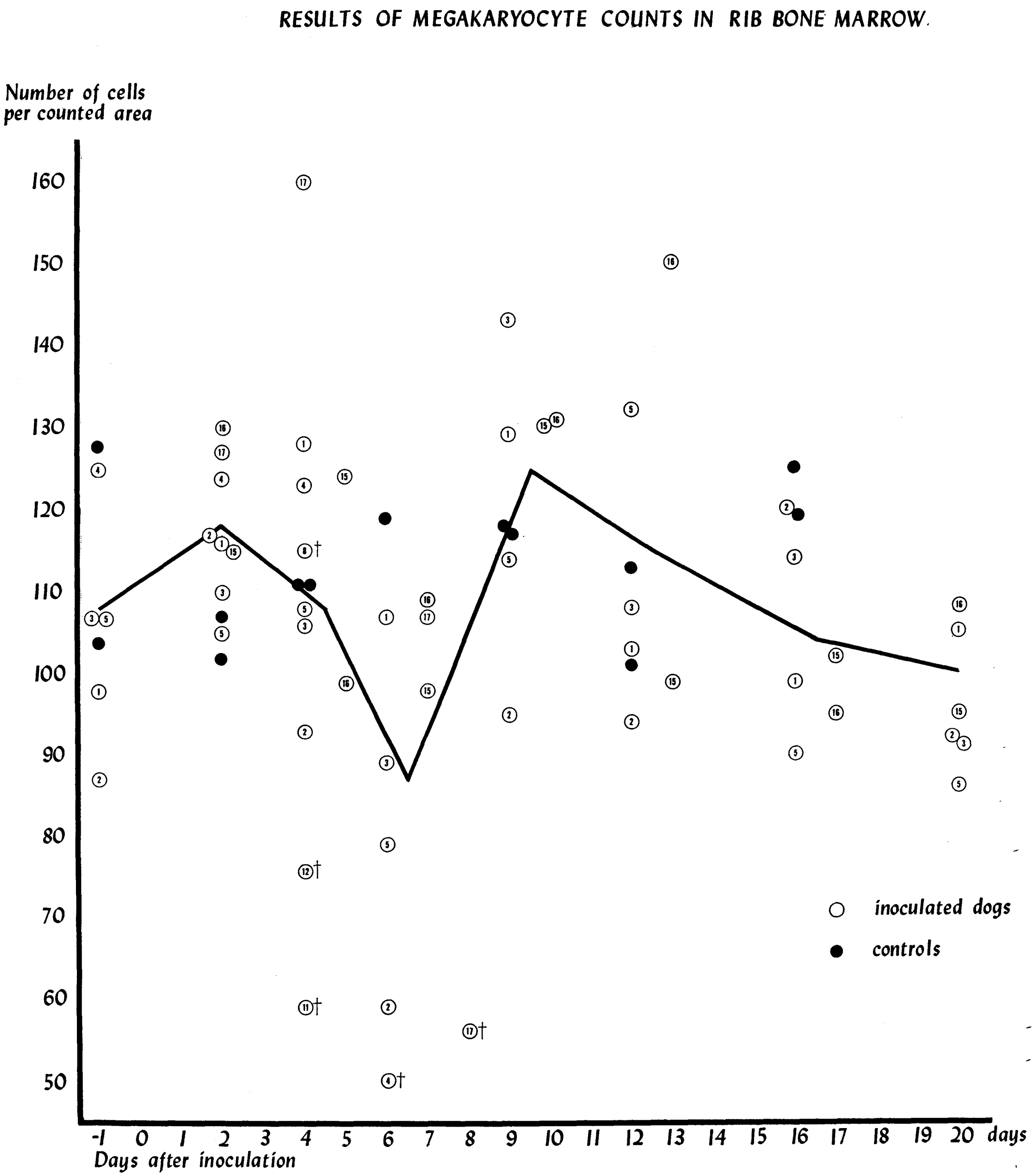


Fig. 3. Number of megakaryocytes in bone-marrow sections from dogs with experimental H.c.c.
The continuous line represents average values.

of younger small thrombocyte-producing megakaryocytes. In the dogs that had died, all the megakaryocytes showed degeneration of varying degree (figs. 5 and 7).

9—10 days after inoculation: The degenerative changes were insignificant. Many degenerating cells consisted of naked pycnotic nuclear rests. Apart from this, the picture was dominated by young thrombocyte-producing cells. There was an absolute increase in the total number of megakaryocytes (fig. 6).

12—13 days after inoculation: There was very active thrombocytopoiesis. The megakaryocytes appeared to be slightly more mature than at the previous examination and most of them were relatively large, with wide cytoplasmic zones.

16—17 days after inoculation: The mature thrombocyte-forming megakaryocytes predominated. Degenerating cells occurred in slightly larger numbers than at the previous examination.

20 days after inoculation: The picture was on the whole identical with that seen at examination before inoculation.

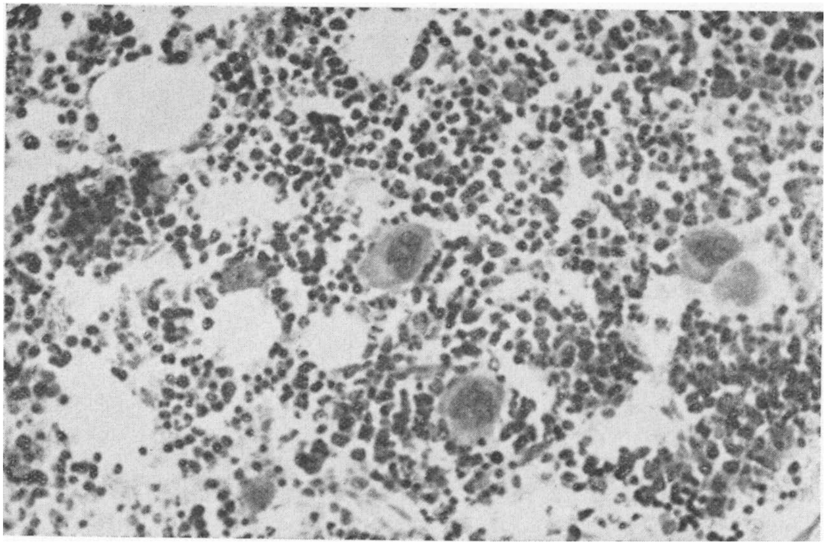
The dogs used as controls showed no great deviations with respect to the megakaryocytes.

DISCUSSION

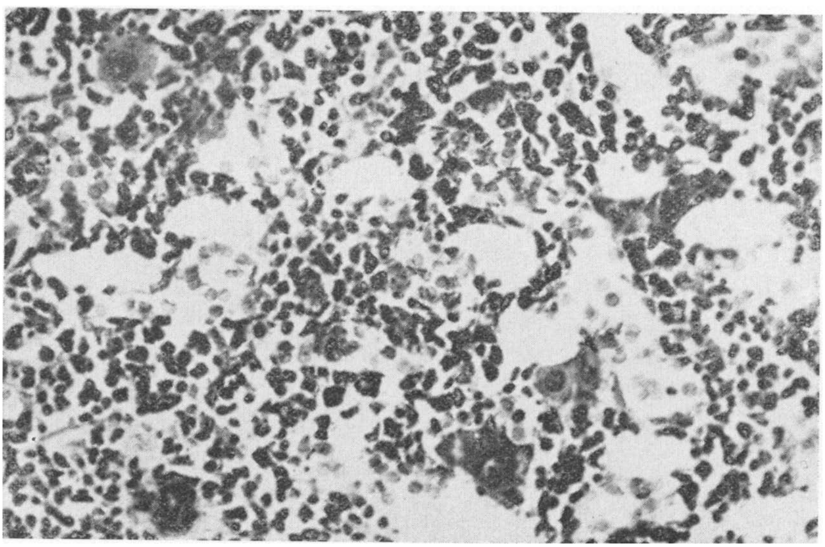
A haemorrhagic tendency in thrombocytopenia has been clearly demonstrated (*Stefanini & Dameshek* 1955, *Ratnoff* 1960, *Nilsson* 1961, *Nordén* 1962). The thrombocytopenia established here, together with the endothelial-cell damage described by *Lindblad & Björkman* (1964), would explain the well-documented haemorrhagic tendency present in H.c.c. A lowering of the prothrombin index can influence the tendency to bleed, but according to *Quick* (1942), the haemorrhagic tendency does not appear until the prothrombin concentration has fallen to less than 20 % of the normal.

The longest clotting-times were measured on day 5 after inoculation, being 37 and 20 minutes. Prothrombin index on this day was 81 and 82, respectively. Thus the longest clotting-times do not correspond to the lowest values for prothrombin index.

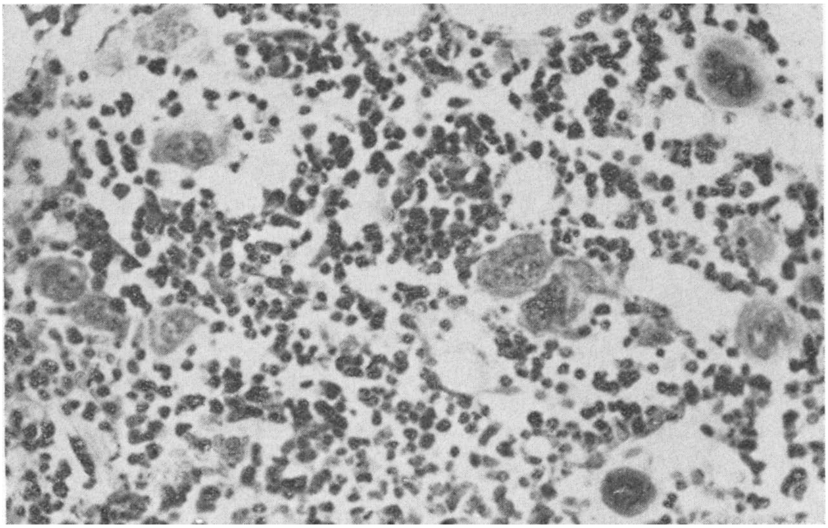
Fibrinogen determinations were not made in this study, but the rise of the erythrocyte-sedimentation rate (table 3) which occurred, argues against a decrease of fibrinogen. An increase of the fibrinogen and globulin fractions in the plasma is con-



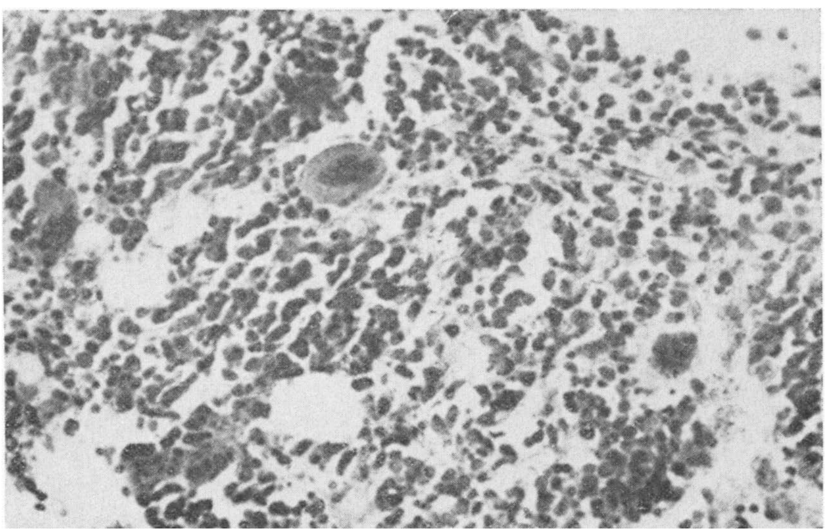
F i g. 4. Rib-marrow section from dog no. 3 before inoculation. Azure eosinate stain (× 300).



F i g. 5. Rib-marrow section from dog no. 3 on the 6th post-inoculation day. The dog was severely ill. Note the degenerating megakaryocytes. Azure eosinate stain (× 300).



F i g. 6. Rib-marrow section from dog no. 3 on the 9th post-inoculation day. Most of the megakaryocytes consist of young thrombocyte-producing cells. Azure eosinate stain (× 300).



F i g. 7. Rib-marrow section from dog no. 17 on the 7th post-inoculation day and about 12 hours before death. All the megakaryocytes are degenerating. Azure eosinate stain (× 300).

Table 3. Erythrocyte-sedimentation rates in dogs with experimentally produced H.c.c. (The sedimentation rate is corrected with P.C.V.)

Dog no.	Days after inoculation						
	0	1	3		5	7	9
8	0	0	88	died			
9	12	0	88		60	80	0
10	21	0	88		90	20	0
11	22	24	84	died			
12	33	22	54	died			

sidered to be the cause of a raised erythrocyte-sedimentation rate (*Fåhraeus* 1921, *Broom* 1937).

In association with experimentally induced thrombocyte depletion the number of thrombocytes falls immediately in the circulating blood of dogs. There are evidently no reserves of thrombocytes (*Craddock et al.* 1955, *Matter et al.* 1960). The decrease in the number of thrombocytes per mm^3 of blood which was observed in this study, reaches a minimum value within 4—7 days after the inoculation. The mortality is highest on these days (*Rubarth* 1947, *Baker et al.* 1950, and personal observations). A question of great interest is whether the decrease is attributable to increased consumption or to reduced production. No data on the life-span of the thrombocytes in the circulation blood of dogs can be found in the available literature. For rats, a survival of 4.5 days has been reported (*Odell & Anderson* 1959) and for man the statements vary between 9—11 days (*Aas & Gardner* 1958) and 8—9 days (*Leeksma & Cohen* 1956). It will be seen from fig. 3, which shows the changes in the number of megakaryocytes, that a reduction of the total number occurs within 6—7 days of the inoculation and that a peak on the 9th—10th day is followed by gradual return to normal. The continuous lines which represent the means for the number of megakaryocytes in the bone-marrow and the total number of thrombocytes per mm^3 of blood reach their minimum values at approximately the same time. This fact, together with the described qualitative changes, argues in favour of a decrease of the thrombocyte production. The question whether the thrombocytes are exposed to a direct attack by virus or are destroyed as a result of the established endothelial damage is difficult to answer. Blood-plate thrombi were not demonstrated in the intravital microvascular study (*Lindblad, Brånemark & Lindström* 1964). The circulatory disturbances that were demonstrated in the said study led to complete cessation of flow in some capillaries. The flow-rate in the sinusoidal system of the bone-marrow is low normally (*Brånemark* 1959). A generalized circulatory disorder can therefore be presumed to cause a metabolic disturbance, particularly in this organ. The observed endothelial-cell damage (*Lindblad & Björkman* 1964) and the associated change in permeability would accentuate this metabolic disturbance.

Evidence for a direct action of H.c.c. virus on the megakaryocytes cannot be found in the available literature. However, a direct virus attack could presumably produce changes in the thrombocytes and megakaryocytes similar to those described here.

The thrombocytes can also act as haptens to the virus with resulting development of antibodies and thrombocytopenia. The established prolongation of clotting-time can be caused by several factors. The decrease in the number of thrombocytes and the change in prothrombin index may play a part, but the question whether other clotting-factors are involved requires further investigations.

REFERENCES

Aas, K. A. & Gardner, F. H.: Survival of platelets labeled with chromium^{51}. J. Clin. Invest. 1958, *37*, 1257—1268.

Baker, J. A., Jensen, H. E. & Witter, R. E.: Canine infectious hepatitis-Fox encephalitis. J. Am. vet. med. Ass. 1954, *124*, 214—216.

Berman, L., Axelrod, A. R. & Kumke, E. S.: Estimation of megakaryocyte content of aspirated sternal marrow. Am. J. Clin. Path. 1948, *18*, 898.

Best, C. H. & Taylor, N. B.: The physiological basis of medical practice. The Williams and Wilkins Company, Baltimore 1945.

Broom, J. C.: The correlation between red cell sedimentation rate and plasma proteins. J. Lab. & Clin. Med. 1937, *22*, 998—1000.

Brånemark, P-I.: Vital microscopy of bone marrow in rabbit. Scand. J. Clin. & Lab. Invest. Suppl. 38, *11*, 1959.

Coffin, D. L. & Cabasso, V. J.: The blood and urine findings in infectious canine hepatitis. Am. J. Vet. Res. 19553, *14*, 254—259.

Craddock, C. G., Adams, W. S., Perry, S. & Lawrence, J. S.: The dynamics of platelet production as studied by a depletion technique in normal and irradiated dogs. J. Lab. & Clin. Med. 1955, *45*, 906—919.

Fåhraeus, R.: The suspension stability of the blood. Physiol. Rev. 1929, *9*, 241—274.

Gillespie, J. H.: Clinical features of infectious canine hepatitis. Proc. 89th Ann. Meet. Am. vet. med. Ass. 1952, 224—225.

Hodgman, S. F. J. & Larin, N. M.: Diagnosis of canine virus hepatitis (Rubarth's disease). Vet. Rec. 1953, *65*, 447—450.

Leeksma, C. H. W. & Cohen, J. A.: Determination of the life span of human blood platelets using labeled diisopropylfluorophosphonate. J. Clin. Invest. 1956, *35*, 964—969.

Lehman, J.: Protrombinbestämning i kliniken. En ny modifikation av Quicks metod. Nord. med. 1941, *12*, 3192—3197.

Lillie, R. D.: Histopathologic Technic. Philadelphia. 1948.

Lindblad, G. & Persson, F.: Transaminase and transferase activities in blood plasma of dogs with experimentally produced Hepatitis contagiosa canis (H.c.c.). Acta vet. scand. 1962, *3*, 378—390.

Lindblad, G., Brånemark, P. I. & Lindström, J.: Capillary form and function in experimental Hepatitis contagiosa canis. An intravital microvascular study. Acta vet. scand. 1964, *5*, 384—393.

Matter, M., Hartman, J. R., Kautz, J., DeMarsh, Q. B. & Finch, C. A.: A study of thrombopoiesis in induced acute thrombocytopenia. Blood, 1960, *15*, 174—185.

Nilsson, I. M.: Blödningstillståndens klinik och terapi. Svenska Läkartidn. 1961, *58*, 1997—2020.

Nordén, Å.: Trombocyter och trombocytopeni. Svenska Läkartidn. 1962, *59*, 1905—1942.

Odell, T. T. & Andersson, B.: Production and lifespan of platelets in the kinetics of cellular proliferation. Grune and Stratton, New York, 1959, 278—280.

Poppensiek, G. C.: Virus diseases of dogs. With special reference to infectious hepatitis. Vet. Med. 1952, *47*, 282—287.

Quick, A. J.: Haemorrhagic diseases and the physiology of hemostasis. Springfield, Il. Charles C. Thomas, 1942.

Ratnoff, O. D.: Bleeding syndromes. A clinical manual. Charles C. Thomas. Publisher, Springfield, Ill. U.S.A. 1960.

Rubarth, S.: An acute virus disease with liver lesions in dogs. (Hepatitis contagiosa canis). Acta path. microbiol. scand. Suppl. 69, 1947.

Smith, D. L. T.: Observations on infectious canine hepatitis. Am. J. vet. Res. 1951, *12*, 38—43.

Stefanini, M. & Dameshek, W.: The haemorrhagic disorders. Grune and Stratton, New York, London, 1955.

Thorell, C. B.: En metod att räkna trombocyter i hundblod. Nord. Vet. Med. 1962, *14*, 724—727.

SUMMARY

This paper reports a study of total thrombocyte counts, prothrombin index, clotting-times, and qualitative and quantitative determinations of the megakaryocytes in dogs with experimentally produced Hepatitis contagiosa canis. The megakaryocytes were studied in rib-marrow sections. A marked decrease was noted in the total number of thrombocytes down to less than 20,000 platelets per mm^3 within 4—7 days of the inoculation. The prothrombin index changed

very little. The haemorrhagic tendency in Hepatitis contagiosa canis is considered to be due to the thrombocytopenia in combination with vascular damage. A decrease in the number of megakaryocytes and qualitative changes in these cells were demonstrated. A prolongation of the clotting-time was established and its causes are discussed.

ZUSAMMENFASSUNG

Megakaryozyten, Thrombozyten und Blutgerinnungszeit bei Hunden mit experimenteller Hepatitis contagiosa canis.

An Hunden mit experimenteller H.c.c. wurden die Veränderungen in der Gesamtanzahl der Thrombozyten, des Protrombinindexes, der Blutgerinnungszeit als auch die qualitativen und quantitativen Veränderungen im Megakaryozytenbild untersucht. Das letztgenannte wurde durch Biopsie der Rippen untersucht. Eine ausgeprägte Senkung der gesamten Thrombozyten bis zu Werten oft unter 20.000 Thombozyten/mm^3 4—7 Tage nach der Inokulation, wurde festgestellt. Der Protrombinindex wurde kaum verändert. Es wird angenommen, dass die Neigung zu Blutungen bei der H.c.c. von der Thrombozytopenie samt Blutgefässschädigungen abhängig ist. Die Verminderung der Zahl der Megakaryozyten als auch die bei ihnen festgestellte qualitative Veränderungen werden angegeben. Einer Verlängerung der Blutgerinnungszeit wurde festgestellt und ihre Ursachen besprochen.

SAMMANFATTNING

Megakaryocyter, trombocyter och blodkoagulationstid hos hundar med experimentell Hepatitis contagiosa canis.

På hundar med experimentellt framkallad H.c.c. har förändringar av totalantalet trombocyter, protrombinindex, blodkoagulationstider samt kvalitativa och kvantitativa förändringar i megakaryocytbilden följts. De senare har studerats på snitt från rebensbiopsier. En markant nedgång av totalantalet trombocyter till värden ofta understigande 20.000 trombocyter/mm^3 4—7 dagar efter inokulationen har konstateras. Protrombinindex förändras lindrigt. Blödningsbenägenheten vid H.c.c. anses bero på trombocytopenin i kombination med kärlskada. Minskning av antalet megakaryocyter samt hos dem iakttagbara kvalitativa förändringar redovisas. En förlängning av blodets koagulationstid har konstaterats och dess orsaker diskuteras.

(Received February 8. 1964).