Ontogeny of the granulocyte/macrophage progenitor cell (GM-CFC) pools in the beagle

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SUMMARY

The pattern of development of the granulocyte/macrophage progenitor cell (GM-CFC) pools in the course of canine ontogeny was studied by means of the agar culture technique. Colony formation was stimulated by colony stimulating activity (CSA) in serum from lethally irradiated dogs in combination with erythrocyte-depleted peripheral blood leukocytes from normal adult dogs. The colonies thus obtained in cultures from the different organs were in general large (estimated maximum 50000 cells) and consisted predominantly of mononucleated macrophages, suggesting that, in these studies, a progenitor cell with high proliferative potential (HPP-CFC) has been monitored.

In the yolk sac, a transitory GM-CFC pool became established between day 23 and day 48 of gestation, reaching maximum numbers of approximately 41×10^3 per organ on days 36/37. At the same time the GM-CFC concentration in blood collected from the heart also reached a maximum of about 31×10^3/ml, indicating its carrier function for the migration of GM-CFC. In the liver a quasi-exponential increase in the GM-CFC numbers took place between days 36/37 and days 57 to 59 when a total of about $15 \cdot 2 \times 10^6$ was found but thereafter and up to day 4 *post partum* the GM-CFC numbers decreased by almost two orders of magnitude. A continuous increase in the GM-CFC numbers was found in the spleen between day 42 of gestation and day 4 *post partum* when a maximum of $5 \cdot 1 \times 10^6$ to $8 \cdot 7 \times 10^6$ was reached. In contrast to the GM-CFC numbers in the liver, the splenic GM-CFC dropped only by 50 % of peak values when the dogs reached adulthood. The bone marrow always had the highest incidence of GM-CFC, the concentration per 10^6 cells being $18 \cdot 7 \times 10^3 / 10^6$ cells on days 45/46, the earliest time point at which cultures could be set up. The absolute GM-CFC numbers in the two femora increased continuously between days 45/46 and day 4 *post partum* in parallel with the growth of the bones. In the thymus a relatively small population of GM-CFC developed between days 42 and 48 of gestation that was kept quite constant at average numbers between 13×10^3 and 30×10^3 up to day 4 *post partum*.

INTRODUCTION

In the last two decades, the dog has been intensively used in the field of pre-clinical studies, especially with regard to the response of the lympho-haematopoietic system to different forms of disturbance and their treatment modalities (Shifrine & Wilson, 1980).

Among the various techniques available for the study of early haemopoietic cells, procedures that permit the quantitative and qualitative characterization of the granulocyte/macrophage progenitor cell GM-CFC compartments have been intensively applied (Wilson & Shifrine, 1980). According to the results obtained from such studies, three major pools of GM-CFC can be defined in the adult dog comprising the bone marrow, the blood (Debelak-Fehir, Catchatourian & Epstein, 1975; Kovacs, Bruch & Fliedner, 1976), and the spleen, their quantitative relationship being of the order of $100:0\cdot1:1$, respectively (Nothdurft, Calvo, Fliedner & Schnappauf, 1980; Nothdurft, Steinbach, Ross & Fliedner, 1982).

In contrast to this situation in the adult, virtually no information was available up to 1981 with respect to the developmental pattern and the definite establishment of the GM-CFC pools in different organs in the course of canine ontogeny. Some results from current studies performed by two different groups and directed to various aspects have recently been published (Nothdurft, Braasch, Calvo, Carbonell, Grilli & Fliedner, 1981; Klein et al. 1983). It is the purpose of this paper to report on the follow-up of the quantitative changes of the GM-CFC in five different organs and the blood of the beagle during the whole period of gestation.

MATERIALS AND METHODS

Animals and general experimental design

The pregnant bitches were from four different colonies. They had been date-mated or kept together with the male on three alternative days within a 5-day period. The foetuses were delivered at a given time by Caesarean section. Either all the foetuses were collected by a complete hysterectomy or the pups located within one horn each, were obtained by partial hysterectomy on two successive occasions. The foetuses were collected at gestation ages between day 20 and day 59. Because of the retrospective method for the determination of the definitive age (see next paragraph) and the 5-day interval allowed for mating in some of the cases, no exact grouping for more than 1 litter could be obtained at certain days of age. However, a cumulation of between two and four litters at a certain age was obtained for days 36/37, days 45/46, days 49–51, days 54–56 and days 57–59. From each litter two or three foetuses were studied. The organs and tissues studied for GM-CFC were yolk sac, peripheral blood, liver, spleen, bone marrow and thymus. In addition, two 4-day-old pups were studied.

Calculation of gestation age

For 12 litters out of a total of 19 studied the exact date of conception and thus the exact number of days between conception and surgical delivery was known.

When the crown–rump length of these pups was plotted against the gestation age the relationship was found to be quite similar to the comprehensive set of data published by Evans (1979). Thus a common weighted curve was fitted to all the data and for all uncertain cases the most probable gestation age interpolated from this curve on the basis of the measured crown–rump length.

Sampling and preparation of organs

First the yolk sac was cut away from the foetus, reduced to small pieces and put into a tube with serum-free medium, type S-MEM (GIBCO EUROPE, U.K.).

After opening of the chest wall of the foetus, blood was collected by heart puncture into a heparinized syringe. A small aliquot of the sample was used for performing nucleated blood cell counts and for the preparation of smears. The volume of the remainder was determined, then transferred into a tube and washed.

The liver, spleen and thymus were excised, cleaned from adherent tissue and weighed immediately. If the total organ weight did not exceed about $0.4\,g$, the whole organ was used for further preparations as was also the yolk sac independent of its size. Otherwise several small samples of tissue were collected from different regions of each organ, pooled for each foetus and again weighed exactly, resulting in total amounts of between 0.25 and $0.4\,g$ each. To obtain bone marrow, the two femora were freed of the adherent tissue, both tips of the bones were cut off and the marrow was flushed out into a tube with S-MEM by means of a syringe that was connected to the bone by a small tube.

The pieces of the yolk sac, the specimens from liver, spleen and thymus were then carefully treated with glass homogenizers containing ice-cold S-MEM for preparing cell suspensions. Macroscopic aggregates were eliminated by a short-term sedimentation for $2\,min$ at $1\,g$.

The cells prepared from the blood, the bone marrow, the liver, the spleen, the thymus and the yolk sac were (without any separation) suspended in $15\,ml$ S-MEM, centrifuged and resuspended in an appropriate amount of complete culture medium containing 20% horse serum to a known complete volume. The concentration of nucleated cells (all types) per ml cell suspension was determined using a Neubauer chamber. Since the volume of each cell suspension was known, the absolute cell yield obtained from individual organs could be determined directly, or calculated indirectly by means of the cell numbers obtained from the quantified samples from the different organs and the total weight of the respective organ.

Preparation of agar cultures for GM-CFC determinations

The cell suspensions prepared from the different organs and the heart blood were kept on ice. A standard method was applied for the preparation of agar cultures (Nothdurft *et al.* 1980). The cells were plated as $1\,ml$ suspensions in

supplemented culture medium containing 20 % horse serum and 0·3 % of agar (DIFCO Laboratories, Detroit, USA). As a source of colony-stimulating activity (CSA), 0·3 ml of serum obtained from lethally irradiated dogs was added to each plate. For enhancement of colony formation, blood leukocytes obtained from a normal adult dog and irradiated with 12 Gy to kill proliferating cells were added at numbers of $1·6×10^6$ polymorphonucleated granulocytes (PMNs) to each plate. Under routine conditions the cell suspensions from different organs were plated at varying numbers that, according to a series of pilot studies, would result in colony numbers within the linear part of the curve relating cell dose to colony number. The nucleated cell numbers inoculated per dish were $0·01×10^6$ for bone marrow, $0·025×10^6$ for spleen, $0·05×10^6$ and $0·1×10^6$ for blood, $0·1×10^6$ for liver, $0·1×10^6$ and $0·2×10^6$ for yolk sac and $0·2×10^6$ for the thymus. The cultures were incubated for 7 days and aggregates of more than 40 cells were scored as colonies. For cytological studies 40 to 50 colonies were picked off sequentially from the cultures, fixed with methanol and stained with haematoxylin–eosin.

RESULTS

Colony growth and differentiation

The cultures stimulated by both serum CSA and irradiated leukocytes provided linear dose-response curves for colonies versus cells plated down to the range of $0·01×10^6$ cells plated for bone marrow and $0·025×10^6$ to $0·1×10^6$ for cell suspensions from the other organs. The colonies were of relatively compact structure and of considerable size having between 100 cells and a roughly estimated number of about 50 000 or more (Fig. 1). From cytological examinations it became evident that among the colonies harvested from the cultures, more than half contained 60 % to 80 % or large macrophages or were pure macrophage aggregates, independent of the source of the cells and the foetal age. Colonies with more than 80 % neutrophilic granulocytes, or pure granulocyte colonies, were relatively rare, mainly of the order of 0 % to 11 % of all clones in different experiments.

It is noteworthy that in some cultures from yolk sac, liver and blood erythroid aggregates could be identified on day 7 of culture, most of them lying close to the large and densely packed macrophage/granulocyte colonies (Fig. 2). These erythroid aggregates were uniform or multicentric (like the regular erythroid bursts), contained a total of 50 to 500 cells and always showed a close association between erythroblasts and large macrophages within the aggregates. A relatively high number of such erythroid colonies was found in cultures from the yolk sac and liver of 36-day-old foetuses. In cultures from the yolk sac and liver of 42- and 46-day-old foetuses, such erythroid colonies were rare or absent.

In some experiments performed concomitantly with the regular studies, cultures from the different organs were stimulated by serum CSA only, without the
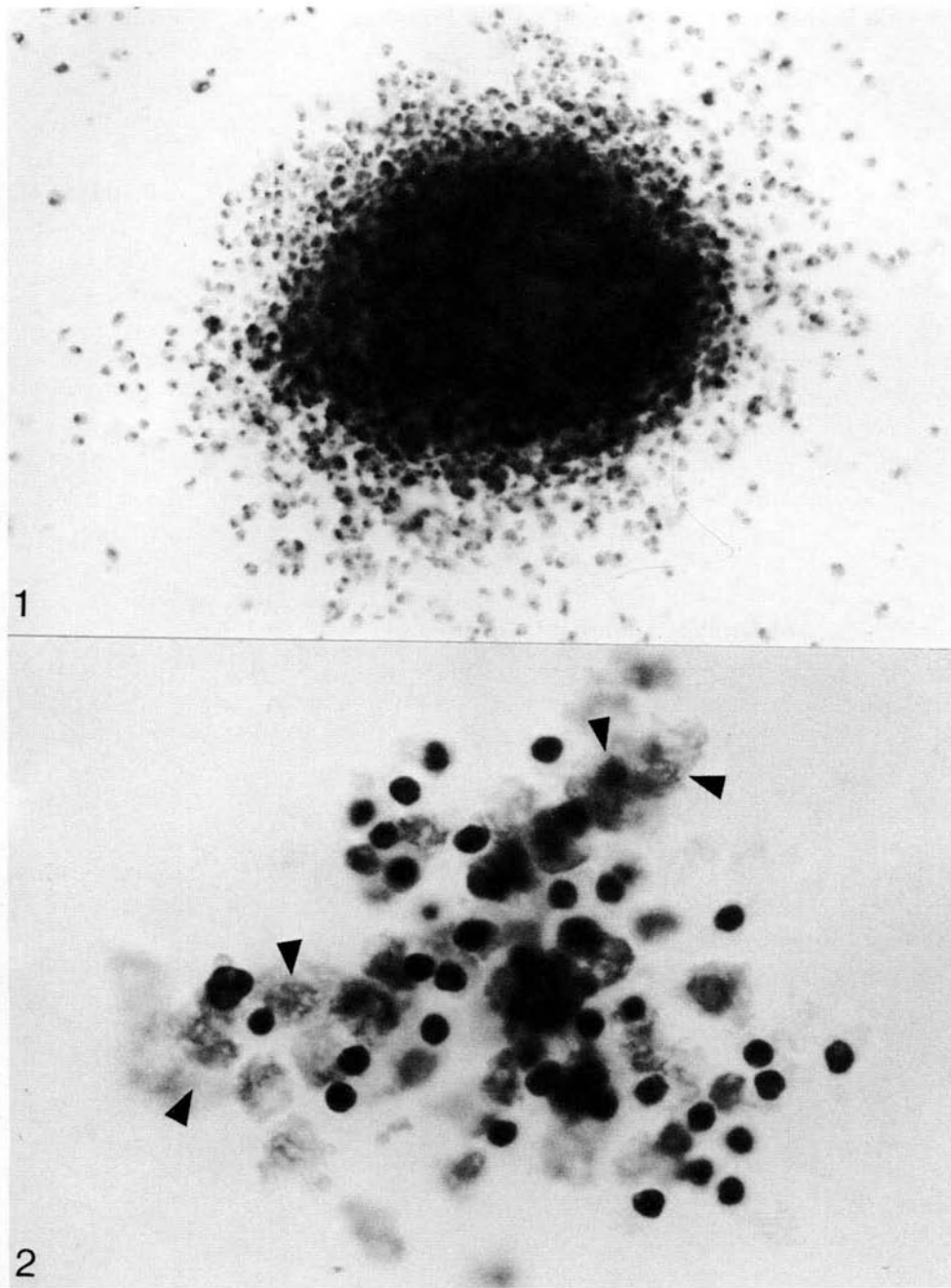
Fig. 1. Large compact macrophage colony grown in a culture of canine liver cells at day 36 of gestation (×200).

Fig. 2. A small colony of erythroblasts intermixed with large macrophages (arrowheads) in a culture of canine yolk sac cells at day 36 of gestation (×1250).

addition of irradiated leukocytes. Under these circumstances, considerably lower numbers of colonies were obtained and the colonies were much smaller than those described above. However, at least for foetal liver there was a tendency for increased numbers of those colonies in which more than half the cells were neutrophilic granulocytes.

Concentration of GM-CFC in different tissues and organs

Up to a gestation age of 36 days, when the foetuses reached a crown–rump length of an average value of 3·8 cm, three tissues reached a developmental stage that permitted quantitative GM-CFC determinations, i.e. the yolk sac, the liver and the blood (Fig. 3). The first time GM-CFC were determined was for embryonic 'anlagen' in total, with an average weight of 0·13 g, corresponding to an age of about 20 days. The value of three GM-CFC/10^6 embryonic cells obtained thus probably represents progenitor cells from the developing yolk sac. Starting from day 23, when the yolk sac could be separated from the embryo, up to day 59 between 120 and 700 GM-CFC/10^6 cells were found. During the last few days of foetal life, the yolk sac became atrophic.

Day 36 was the earliest time that the heart could be manipulated so that 'some' blood for performing agar cultures could be obtained. For this purpose the heart, while kept in culture medium, was cut into four pieces and the cells presumed to be present in its cavities washed into the medium. The total cell yield averaged 1·57×10^6 per organ, concentrated in a volume far too small to be determined but
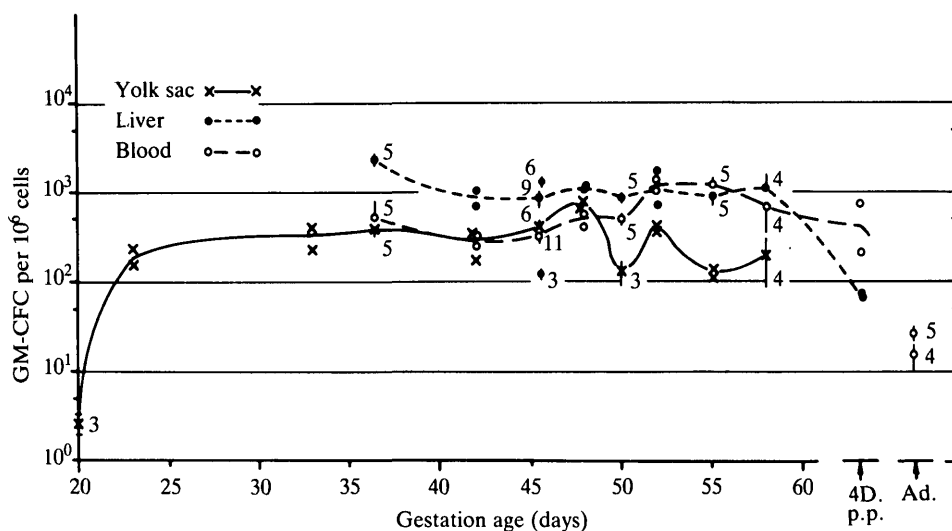

Fig. 3. Incidence of GM-CFC per 10^6 cells in the yolk sac, liver and blood of canine foetuses between days 20 and 57 to 59 of gestation. Values are also shown for two pups on day 4 *post partum* and two collectives of adult dogs. The points plotted are mean values x̄ (±1 s.e.m.) obtained from the data of 3 to 11 foetuses (indexed numbers) or individual values obtained from 2 foetuses at certain dates (without numbers).

very probably amounting to not more than 0·01 ml at the most. The average GM-CFC concentration was $541/10^6$ cells. Thereafter, when considerable amounts of blood could be collected from the heart allowing for the determination of the exact volume, the GM-CFC concentration was found to be higher than $300/10^6$ cells up to days 45/46, rising to about $1 \times 10^3/10^6$ cells between days 52 and 56 and declining again by day 4 *post partum*. As is shown in Fig. 3, the GM-CFC concentration for two different collectives of adult normal dogs determined at two different times was $15/10^6$ or $26/10^6$ nucleated blood cells, respectively (Nothdurft, 1980; Nothdurft *et al.* 1980).

The liver weight in the 36- to 37-day-old foetuses averaged 0·366 g. Thus this organ was already in a well-developed state when the first GM-CFC determinations were performed. This was the age when the highest GM-CFC concentration was found within the whole gestation period, amounting to $2·3 \times 10^3/10^6$ cells on the average. Thereafter, the average values remained quite stable at a level of about $1 \times 10^3/10^6$ cells but showed a dramatic decline from day 59 of gestation to day 4 *post partum*, when the index was $70/10^6$ cells. Especially in the case of foetal liver, considerable variations in the GM-CFC concentrations were observed between different litters. By far the most conspicuous differences are represented in Fig. 3 for the group(s) at an age of 45/46 days. Among the four litters studied, the individual GM-CFC concentrations ranged from $547/10^6$ cells to $1·7 \times 10^3/10^6$ cells in six foetuses from three litters, whereas in the three foetuses from the fourth litter the individual values were comparatively low but more uniform between $112/10^6$ cells and $127/10^6$ cells. According to this special situation three different values are depicted in Fig. 3: the average value obtained for the whole collective (n = 9), and the average values for the two 'subgroups' (n = 3 and n = 6, respectively).

The other three organs studied were not available for GM-CFC determinations before day 42 (Fig. 4). On that day the spleen weight in the two respective foetuses studied was 8·5 mg and 10 mg. However, in these spleens the GM-CFC concentration was extremely high giving values of about $3·4 \times 10^3$ and $6·5 \times 10^3$ per 10^6 cells. Thereafter the indices remained quite stable at a level between 2×10^3 and 3×10^3 per 10^6 cells up to days 57–59, and even at 4 days *post partum* the GM-CFC values were still at the upper level of the range existing during foetal development. The splenic GM-CFC concentration in adult dogs determined in previous studies averaged 129 GM-CFC/10^6 cells (Nothdurft *et al.* 1980) and is shown in Fig. 4 for comparison.

The GM-CFC in the bone marrow of the two femora could be determined the first time on days 45/46 of gestation when a total of between $0·28 \times 10^6$ and $1·35 \times 10^6$ cells (average value $0·63 \times 10^6$ cells) could be collected from the cavities of these two long bones. On this day, the average GM-CFC concentration was $18·7 \times 10^3/10^6$ nucleated cells and remained at this level up to day 50. From day 52 of gestation up to the fourth day *post partum* the average values were considerably lower but remained stable at a level between about 7×10^3 and 9×10^3 per 10^6
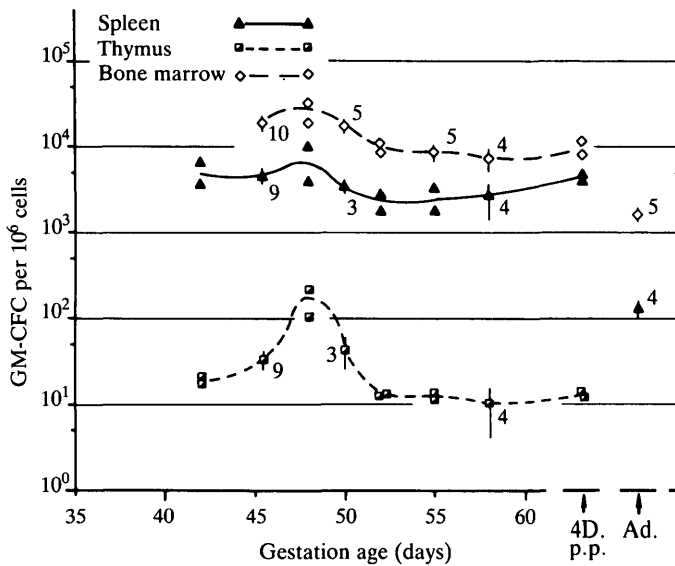
Fig. 4. Incidence of GM-CFC per 10^6 cells in the spleen, the thymus and the bone marrow of canine foetuses between days 42 and 57 to 59 of gestation. Values are also shown for two pups on day 4 *post partum* and a collective of adult dogs. (For further explanations see legend to Fig. 3.)

cells. Thus, over the whole period of gestation the GM-CFC concentration in the bone marrow was the highest compared to all the other organs, i.e. about $\times 10$ higher than in foetal liver and between $\times 3$ and $\times 5$ higher than in the foetal spleen. In the bone marrow of five adult normal dogs the average GM-CFC concentration is $1 \cdot 6 \times 10^3 / 10^6$ cells (Fig. 4).

The GM-CFC concentration in the thymus could first be determined at a foetal age of 42 days, when the organ weight from two foetuses was 60 mg and 80 mg. The GM-CFC concentrations were always low, mostly ranging from 10 to $41/10^6$ cells. On day 48 a considerable but transitory increase to 100 and 200 GM-CFC/10^6 cells could be observed.

Absolute numbers of GM-CFC in different tissues and organs

The changes in the absolute GM-CFC numbers in the three tissues in which progenitor cell determinations were possible within the first 36 days of gestation, i.e. yolk sac, heart blood and liver, are shown in Fig. 5. For the embryonic 'anlagen' on day 20, an average total of 28 GM-CFC (range 8–39) was found among 6×10^6 to 13×10^6 cells obtained from the embryos. From day 23 on, when GM-CFC could be selectively determined for the yolk sac, they showed a sharp increase up to day 36 when their number amounted to about 41×10^3 per organ. Thereafter a slight decrease took place up to day 48 to 11×10^3 and 21×10^3 GM-CFC. Beyond day 48 a much faster reduction in the pool size was observed up to days 57–59 when the GM-CFC numbers per organ just reached a value of 321.

The values for the GM-CFC in the blood depicted for day 36 should be viewed as only crude estimates, since the volume of blood could not be determined. However, based on an assumed volume of 0·01 ml collected from the heart a number of 31×10^3 GM-CFC/ml blood was calculated. Since the value of 0·01 ml is most probably an overestimate, the blood GM-CFC concentration may *de facto* be even higher than the calculated value. From days 36/37 on there was a slow but steady decrease in the blood GM-CFC concentration up to day 4 *post partum* when it reached approximately 2×10^3/ml or 7×10^3/ml in the two dogs studied. In adult dogs the GM-CFC concentration has been found to vary between 120/ml and 300/ml for two different collectives (Northdurft, 1980; Northdurft *et al.* 1980).

In the liver a nearly exponential increase in the GM-CFC numbers was observed over the whole period of gestation. A peak average value of approximately $15·2 \times 10^6$ GM-CFC per organ was reached on days 57–59. There was a dramatic drop within the period up to day 4 *post partum*, when $0·2 \times 10^6$ GM-CFC were found per organ.

A very steep increase in GM-CFC numbers over three orders of magnitude occurred in the spleen (Fig. 6) from about 6×10^3 on day 42 to 5×10^6 and $8·7 \times 10^6$ on day 4 *post partum*. In adult dogs the GM-CFC numbers were found to have stayed at the same level, i.e. an average of $3·2 \times 10^6$ per organ (Nothdurft *et al.*
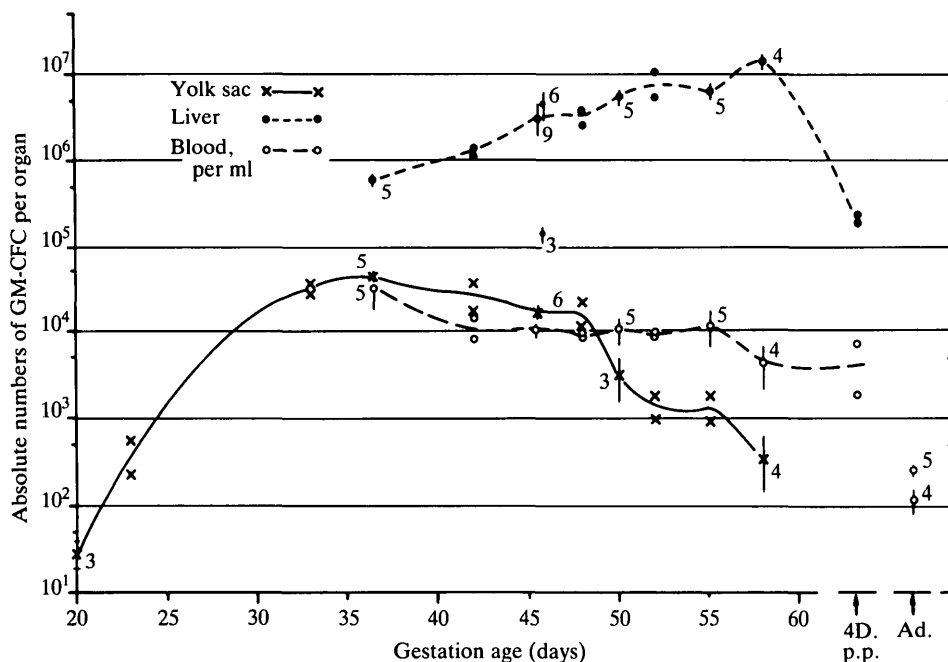

Fig. 5. Absolute numbers of GM-CFC in the yolk sac and liver, and their concentration per ml blood in canine foetuses between days 20 and 57 to 59 of gestation. Values are also shown for two pups on day 4 *post partum* and two collectives of adult dogs. (For further explanations see legend to Fig. 3.)
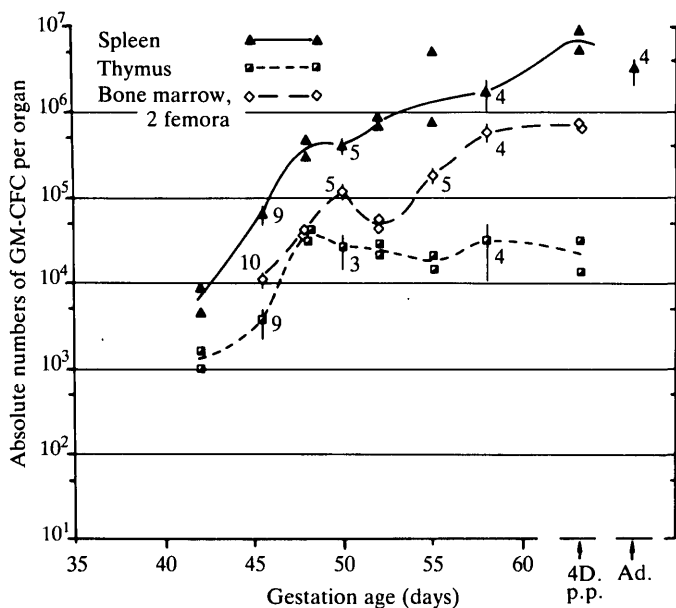
Fig. 6. Absolute number of GM-CFC in the spleen, the thymus and in the bone marrow of the two femora between day 42 and 57 to 59 of gestation. Values are also shown for two pups on day 4 *post partum* and a collective of adult dogs. (For further explanations see legend to Fig. 3.)

1980). A similar increase as for the spleen was found for the total GM-CFC numbers in the bone marrow of the two femora (over nearly two orders of magnitude) between days 45/46 and day 4 *post partum*. On days 45/46 when the bone marrow GM-CFC concentration was $18 \cdot 7 \times 10^3 / 10^6$ cells and thus at its highest point during foetal development, the absolute numbers were $10 \cdot 9 \times 10^3$ per two femora, and $> 6 \cdot 3 \times 10^5$ on day 4 *post partum*. The thymus contained between 1×10^3 and $1 \cdot 6 \times 10^3$ GM-CFC on day 42. Up to day 48 there was an increase to about 31×10^3 and 42×10^3. Thereafter the GM-CFC numbers remained quite stable up to day 4 *post partum* being in the range between 13×10^3 and approximately 30×10^3.

Changes in the blood leukocyte concentration

The presentation of the blood cell changes during foetal development has been restricted to the different subgroups of the leukocytes, because of the special daughter/mother relationship between the granulocytes and monocytes on the one hand and the GM-CFC on the other (Table 1). As can be seen from the data in Table 1, there is an increase in concentration for neutrophilic and eosinophilic granulocytes, monocytes, and lymphocytes from days 45/46 to days 57 to 59. However, except for the monocytes the values at the latter time were found to be smaller than in adult dogs. Lowest values, i.e. only about one fifth of adult values, were found for the neutrophilic granulocytes. In this cell line, there was

Table 1. *White blood cell counts and neutrophilic granulocyte differentials in canine foetuses of different age*

| Age of gestation (days) | Neutrophilic granulocytes | | | | Eosinophilic granulocytes | Monocytes | Lymphocytes |
	$M_1 - M_5$†	juv. + stab	segmented	total			
45/46 (n = 7)	91 ± 60	138 ± 56	58 ± 38	265 ± 135	0	9 ± 9	92 ± 36
49–51 (n = 5)	93 ± 50	150 ± 53	32 ± 32	274 ± 87	0	21 ± 13	952 ± 285
52/53 (n = 9)	29 ± 10	82 ± 17	413 ± 57	524 ± 77	40 ± 10	117 ± 23	425 ± 46
54–56 (n = 6)	9 ± 9	225 ± 42	631 ± 144	866 ± 147	33 ± 23	395 ± 108	1779 ± 328
57–59 (n = 4)	8 ± 8	360 ± 124	683 ± 253	1052 ± 362	215 ± 93	422 ± 94	1774 ± 203
Adult (n = 9)	0	184 ± 35‡	5969 ± 581	6150 ± 559	385 ± 76	366 ± 65	2736 ± 233

Cells per mm³ blood*

* mean values $\bar{x}$ ± s.e.m.; †M_5 = metamyelocyte; ‡no juveniles.

a reduction in the absolute numbers of circulating immature M1 to M5 cells and a far smaller increase in the juveniles (M6) and stab forms (M7) during foetal development when compared to the increase in number of the segmented granulocytes.

DISCUSSION

This paper describes the developmental pattern of the granulocyte/macrophage progenitor cell (GM-CFC) pools in the blood and in five different organs in the course of canine ontogeny between day 20 of gestation and day 4 *post partum*. The GM-CFC changes were determined on the basis of both the concentration of GM-CFC per 10^6 unseparated cells in a certain organ and their total numbers in the different organs or per unit volume of the peripheral blood.

One of the most interesting aspects of these studies is related to the types of colonies obtained in the agar cultures. The colonies grown under the double stimulation by CSA containing serum from irradiated dogs and inactivated leukocytes from adult normal dogs were found to be unusually large when compared to colonies obtained from bone marrow, blood or splenic tissue of adult dogs under similar stimulation. The largest colonies in cultures from foetal tissues were estimated to contain 50000 cells and in general the colonies up to day 4 *post partum* were larger than for adult dogs. Thus, although smaller on the average by one order of magnitude when based on cellularity these foetal canine colonies closely resemble the extremely large colonies formed by macrophage progenitor cells with high proliferative potential (HPP-CFC) in cultures of post-fluorouracil mouse bone marrow (Bradley & Hodgson, 1979). Keeping in mind that in our studies the colonies were assayed after 7 days of culture, whereas the colonies from mouse bone marrow HPP-CFC were analysed after 14 days (McNiece, Bradley, Kriegler & Hodgson, 1982), it seems likely that the colony size may even be under-estimated in our studies.

Similarities between foetal canine GM-CFC and murine HPP-CFC also exist with respect to their differentiation pattern, since the former as well as the latter (Bradley & Hodgson, 1979) give rise to compact aggregates that consist of large mononucleated macrophages. Thus, the foetal canine GM-CFC forming large colonies under the combined stimulation of serum CSA and leukocytes can be assumed to be a relatively primitive progenitor cell that may be closely related to pluripotent stem cells as has been suggested for the murine HPP-CFC by Bradley, Hodgson & Bertoncello (1980).

Since macrophage colonies reaching cell numbers between 1000 and 5000 cells are rare in 7-day agar cultures of adult dog bone marrow or blood cells but become more frequent after 10 days of culture, the foetal canine GM-CFC may either be more sensitive to certain stimulants and thus find more optimal growth conditions than adult dog GM-CFC under standard conditions, or the generation time, especially in their progeny, may be shorter than in the adult status, and/or

the proliferative potential of foetal GM-CFC and their descendents is extremely high – or all these alternatives hold true. At least, this type of cell seems to be really rare in the adult under normal conditions. Indeed, foetal GM-CFC in blood of human foetuses were shown to be more sensitive to placental conditioned medium than GM-CFC from bone marrow of the adult (Linch, Knott, Rodeck & Huehns, 1982), whereas for GM-CFC from adult human blood the contrary was found (Chikkappa, Phillips & Brinson, 1982). Thus, there are good arguments to support the first of the above suggestions. There are also data for human and monkey foetal liver GM-CFC showing that they differ from adult bone marrow GM-CFC with respect to morphological, physical and kinetic parameters (Moore & Williams, 1973).

Erythroid colonies were found in a considerable number of cultures, although no erythropoietin had been added. Roughly half of these colonies were growing in a solitary state, whereas the others were located close to the large macrophage colonies. Thus, it remains open whether or not the association in this latter case is due to the production of a mixed cell colony by a single progenitor cell, as demonstrated by Johnson & Metcalf (1978) for murine foetal liver. However, it is of interest that within such erythroid colonies a certain 'contamination' with macrophages was present. These observations may be indicative of a certain role of macrophages in providing factors that support erythroid colony growth *in vitro* by producing erythropoietin and/or burst promoting activity in the absence of exogenous erythropoietin (Zucali, McDonald, Gruber & Mirand, 1977).

As pointed out, the production of granulocytes *in vitro* under the stimulation by either the combination of serum CSA with peripheral blood cells or serum CSA alone was relatively rare. This compares favourably with the small number of neutrophilic granulocytes in the blood throughout the whole gestation period and their slow increase up to days 57 to 59 of gestation. In human foetuses also granulocytes are not formed in large numbers until shortly before birth (Thomas & Yoffey, 1962). Monocytes, on the other hand, were no more frequent in the foetal canine blood than the neutrophils in spite of their large numbers in the GM-CFC colonies. This suggests either that, if they are produced in relation to the number of *in vitro* GM-CFC and to their *in vitro* proliferative activity, then their release from the different tissues into the blood must be quite limited, or that their *in vivo* production is rather restricted owing to the lack of specific stimulators or, alternatively, the action of inhibitors. Histological findings obtained from different tissues of the foetuses do support this latter assumption, i.e. that the production of macrophages in situ is rather restricted (Calvo, unpubl. observations). A quite similar situation is found in the mouse up to days 13/14 of gestation where, despite the relatively large numbers of GM-CFC in yolk sac and liver, the formation of mature granulocytes and monocytes is minimal in those organs during this period (Metcalf, 1977).

Among the five different organs studied, the yolk sac was the only one that clearly showed a merely transitory establishment of a GM-CFC pool amounting

to a total number of 41×10^3 GM-CFC on days 36/37 that was followed by a continuous decrease to 321 GM-CFC on days 57 to 59. This pattern of change is quite similar to that described for the pluripotent stem cell (CFU-S) and GM-CFC pools in the yolk sac of the murine embryo between 7 and 13 days of age (Moore & Metcalf, 1970). Since for the blood on days 36/37 a high concentration of about 31×10^3 GM-CFC per ml has been estimated and approximately 10×10^3 per ml were determined up to days 54 to 56, the GM-CFC in the blood itself present in the yolk sac could have made some contribution to the yolk sac GM-CFC in total. However, up to day 48 at least, i.e. within the period in which the concentration of GM-CFC per 10^6 cells in the yolk sac was as high or even higher than in the blood, an extra pool of GM-CFC in the yolk sac must have existed. Thereafter, the GM-CFC determined in the yolk sac very probably were mainly those that were present in its blood vessels, since on day 52 and thereafter a maximum of 0·2 ml or at least 0·1 ml of blood contained approximately the same number of GM-CFC as found in the total yolk sac.

In contrast to the yolk sac, the absolute GM-CFC numbers were found to increase continuously in the liver, the spleen and the femoral bone marrow throughout the whole period of gestation. The absolute GM-CFC numbers were first determined on days 36/37 for the liver, day 42 for the spleen and days 45/46 for the bone marrow. In all these organs they were between one and two orders of magnitude higher than would have been expected if contamination by GM-CFC from blood in the vasculature had played a major role. For the changes thereafter, up to day 59, such considerations become completely irrelevant, since the GM-CFC increase exceeds by far the increase in blood volume in these organs. For example the $15·2 \times 10^6$ GM-CFC found in 57- to 59-day foetal liver are equivalent to the number of GM-CFC that are contained within $3·47 \times 10^3$ ml of blood ($4·38 \times 10^3$ per ml) in the same foetuses.

Thus, vastly expanding GM-CFC pools are present in the liver, the spleen and the bone marrow up to day 59 of gestation. A comparable quasi-exponential increase has been documented for GM-CFC and CFU-S in the liver of the mouse between day 10 and 13 of gestation (Moore & Metcalf, 1970), and may also be assumed to exist for the GM-CFC in the liver of the human foetus between days 56 and 112 of gestation on the basis of the incidence per 10^5 cells as determined by Moore & Williams (1973). A quite similar situation for canine foetal liver as found in our studies is described by Klein et al. (1983), insofar as on day 55 the GM-CFC number was found to be twice as high as on day 45. Beyond day 59 of gestation the developmental pattern of the GM-CFC pools in the liver, spleen and bone marrow was different. In the liver a sharp drop over two orders of magnitude occurred just between day 59 of gestation and 4 days post partum and even if the $\sim 200 \times 10^3$ GM-CFC determined at this time are assumed to be still present in the liver of the adult, their numerical contribution to the GM-CFC populations in total is insignificant, since $338 \pm 42 \times 10^6$ GM-CFC have been calculated to be present within the whole bone marrow mass (Nothdurft, 1980).

In the spleen, on the other hand, the GM-CFC pool size attained at the end of gestation is maintained at a fairly constant level over the period immediately after birth up to adulthood (cf. Nothdurft *et al.* 1980).

The data presented in this paper for the very immature bone marrow on days 45/46, showing an extremely high incidence of 18.7×10^3 GM-CFC per 10^6 cells and also a considerable absolute number of about 5×10^3 GM-CFC per femur, contrast with the findings of Klein *et al.* (1983) that 'at day 45 there is very little bone marrow activity'. The major cause for these discrepancies most probably relates to differences between the culture systems applied. The femoral marrow, although not a site of maximal haemopoiesis in the adult dog (Calvo, Fliedner, Herbst & Fache, 1975) has not nearly reached the definite GM-CFC numbers of the adult on day 59 of gestation or day 4 *post partum*. A total of approximately 700×10^3 GM-CFC have been determined for the two femora on day 4 *post partum*, i.e. 350×10^3 were obtained from 1 femur. It is possible, on the other hand, to get a total of 55×10^3 to 77×10^3 GM-CFC from the femur of an adult dog by only one aspiration (Raghavachar, unpubl. observations), thus showing that the femoral GM-CFC pool is still expanding beyond day 4 *post partum*.

The GM-CFC pool in the thymus showed a developmental pattern intermediate between spleen and bone marrow on the one hand and the yolk sac on the other in so far that an initial phase of quasi-exponential growth from day 42 to 48 was followed by a plateau up to day 4 *post partum*. Therefore, it should be discussed to what extent the GM-CFC found in the thymus, especially at these latter times, were due to contamination by GM-CFC present in the microcirculation. On day 4 *post partum* a weight of 0.76 g and 1.33 g was determined for the thymuses of the two pups. Assuming that 1 % of the weight was due to blood content, i.e. 0.008 and 0.013 ml respectively, then a total of 56 or 25 GM-CFC could be expected to stem from the blood. Thus, even if this were an underestimation by one order of magnitude, the 13.6×10^3 and 31.1×10^3 GM-CFC which were found are far in excess of the expected values, showing that indeed a certain GM-CFC pool lodges in this lymphoid organ.

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