

Bovine Erythroid (CFU-E, BFU-E) and Granulocyte-Macrophage Colony Formation in Culture

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Abstract. Progenitor cells of bovine erythrocytes, megakaryocytes, and granulocyte-macrophages were cultured in agar or methylcellulose media. Colony formation was supported by cell-free conditioned medium from short-term cultures of concanavalin A-stimulated bovine peripheral blood leukocytes. Granulocyte-macrophage progenitors proliferated well in both types of semisolid culture media, giving rise to neutrophils (from granulocyte colony-forming units, CFU-G), eosinophils (from eosinophil CFU, CFU-Eo), monocyte-macrophages (from macrophage CFU, CFU-M), and mixed granulocyte-macrophages (from granulocyte-macrophage CFU, CFU-GM). Better growth of megakaryocytes, as well as late (erythroid CFU, CFU-E) and early (erythroid burst-forming units, BFU-E) erythroid progenitors was obtained with methylcellulose. Despite considerable variation in the numbers and types of colonies formed from different aspirates of bovine marrow, the numbers observed were generally comparable to those obtained from human and mouse bone marrow cells. The proliferation of bovine BFU-E and eosinophil progenitors herein described is the first successful report of the culture of these cell types.

Key words: Bovine — Hemopoiesis — Progenitor — BFU-E

Clonal culture techniques for the various hemopoietic bone marrow cells have developed rapidly during the last two decades. After the initial description of *in vitro* colony formation from mouse [1] and human [2] granulocyte-macrophage progenitors, culture of various other hemopoietic stem and progenitor cells has been reported from a number of species including rabbit [3], monkey [4], rat [5], and dog [6]. The formation of bovine macrophage, neutrophil, and erythroid colonies (from erythroid colony-forming units, CFU-E) also has been described, using either the plasma clot technique [7, 8] or methylcellulose medium [9–11].

This communication presents the first description of colony formation from bovine early erythroid (erythroid burst-forming units, BFU-E) and eosinophil progenitors and reports the identification of granulocyte and monocyte-macrophage colonies grown in agar and methylcellulose media. Specific *in situ* stains were used to compare the numbers and types of colonies grown in agar medium from different bone marrow aspirates and to differentiate between the cells of the various lineages.

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Materials and methods

Media and reagents. Ficoll-Paque (specific gravity 1.078 g/cm³) and DEAE-dextran (mol. wt. 500,000) were from Pharmacia (Uppsala, Sweden). Alsever's solution, amphotericin B, pokeweed mitogen (PWM), phytohemagglutinin (PHA), and penicillin-streptomycin (100×) solutions were obtained from GIBCO (Paisley, Scotland, UK). Noble agar was from Difco (East Molesey, UK), and Luxol fast blue from Serva (Heidelberg, FRG). Fetal bovine serum (FBS) and powdered Iscove's and Dulbecco's modified minimum essential media (DMEM) were obtained from Flow Laboratories (Irvine, Scotland, UK), and methylcellulose (MC), deionized bovine serum albumin (BSA), and human erythropoietin (Epo) were from Terry Fox Laboratories (Vancouver, Canada). L-asparagine was from Merck (Darmstadt, FRG), and lipopolysaccharides, concanavalin A (Con A), *p*-diaminodiphenyl benzidine and hematoxylin (Gill No. 3) were supplied by Sigma (Poole, Dorset, UK). Human and bovine recombinant interleukin 2 (IL2) were from Amgen Incorporated (Thousand Oaks, California) and Immunex (Seattle, Washington) respectively, and human recombinant granulocyte-macrophage colony-stimulating factor (hrGM-CSF) was from Amersham (Aylesbury, UK). Murine interleukin 3 (IL3; WEHI 3B) was a gift from Prof. M. Dexter (Manchester, UK).

Animals. A group of 100 healthy calves of either Boran (*Bos indicus*) or N'Dama (*Bos taurus*) breeds were used in the experiments. They were of both sexes and aged between 2 and 6 months.

Bone marrow preparation. An area over the sternal region was shaved and disinfected with 70% (vol/vol) ethanol. Between 1 and 2 ml of marrow were aspirated from the third or fourth sternebra [12] using Salah needles (Downs, Surrey, UK) and 5-ml syringes. Aspirates were immediately transferred into five to ten volumes of complete Iscove's medium containing 100 IU/ml heparin. The marrow suspension was centrifuged on Ficoll-Paque (1000 g, 30 min, 4°C). The cells obtained from the interface between medium and Ficoll-Paque were filtered through a double layer of cotton gauze to remove bone and tissue fragments and washed twice (400 g, 7 min, 4°C) in serum-free Iscove's medium.

Growth factors and conditioned media. Growth factors (IL2, IL3, and hrGM-CSF) were screened in proliferation assays at concentrations between 0.1 and 1000 IU/ml. Post-endotoxin sera were prepared according to Walker et al. [9] by injection of lipopolysaccharides into several animals. Conditioned media were prepared from fetal bovine liver, lung, spleen, and bovine placenta and peripheral blood leukocytes. The latter were obtained from the interface after centrifugation of peripheral blood (in an equal volume of Alsever's solution) on Ficoll-Paque (20°C, 1000 g, 30 min). Cells were suspended to 10⁶/ml in DMEM containing 20% (vol/vol) FBS supplemented with either 2.5 µg Con A, 75 µl PWM solution, or 75 µl PHA solution per ml and incubated at 37°C with high humidity in the presence of 6.5% N₂ and 3% O₂ in N₂. The supernatants were harvested on days 1, 3, 5, and 7 by centrifugation at 1000 g and screened in progenitor assays at concentrations ranging from 0.1% to 10%. Because Con A-stimulated leukocyte-conditioned medium (LCM), harvested on day 5, provided the best growth conditions, a dose-response study was performed according to Metcalf [13], and

Table 1. Average numbers of colonies grown from bovine progenitors (compare *Table 3*) and comparison with previous results from mouse and human

Progenitor cell	Bovine	Murine ^a	Human ^a
CFU-G and CFU-M ^b	270	200	170
CFU-Eo ^b	45	30	30
CFU-Meg ^c	15	30	10
CFU-E ^c	767	200	400
BFU-E ^c	49	30	50

The figures summarize the results obtained from >50 bone marrow aspirates, a minimum of three assays each, per 10⁵ mononuclear cells. Eo, eosinophil; and Meg, megakaryocyte.

^a Data from Metcalf [13].

^b From MC and agar assays.

^c From MC assays.

an LCM concentration of 4% (vol/vol) corresponding to the intermediate region in the linear portion of the sigmoid dose-response curve [13] was used in subsequent assays.

Agar and methylcellulose assays for colony-forming cells. The agar assay was performed as described [13]: five parts of 0.6% (wt/vol) Noble agar were mixed with two parts of FBS previously shown to support colony formation, and three parts of medium prepared by mixing 39 ml of double-distilled water, 1.0 g powdered DMEM, 300 μ l of L-asparagine (6.7 mg/ml in isotonic NaCl), 150 μ l of DEAE-dextran (50 mg/ml in isotonic NaCl), and 490 mg of NaHCO₃. Penicillin-streptomycin was added to give a final concentration of 100 IU/ml and 100 μ g/ml, respectively. The final concentration of agar was 0.3%.

Methylcellulose (MC) medium was prepared by mixing 4 parts of MC (2.3%, wt/vol, in Iscove's medium) and 1 part of 10% (wt/vol) deionized BSA with 3 parts of pretested FBS, 0.1 parts each of 10 mM 2-mercaptoethanol in H₂O, 200 mM L-glutamine in saline, 250 μ g/ml amphotericin B and penicillin-streptomycin (100 \times solutions), plus 1.6 parts comprised of LCM, cell suspension, and EPO in medium with 2% FBS. The final concentrations were 30% (vol/vol) serum, 0.9% (wt/vol) MC, 1% (wt/vol) BSA, 4% (vol/vol) LCM, and 1 IU Epo/ml. Accurate dispensing of the viscous MC was achieved with Combitips (Eppendorf, Hamburg, FRG).

In both the agar and MC assays, 1 ml of soft gel was used per 35-mm plastic petri dish, and each test was carried out in triplicate. Preliminary tests, conducted as described by Iscove and Sieber [14], confirmed the linear relationship between the number of cells initially plated and the number of colonies formed, and, therefore, the number of nucleated cells plated in agar or MC assays was 1 or 2 \times 10⁴/ml and 1 \times 10⁴ to 10⁵/ml, respectively. The cultures were incubated as described above.

Based on time-lapse evaluation studies, colonies derived from CFU-E, CFU-GM, and BFU-E were counted on days 5, 7, and 10, respectively. Cell aggregates and clusters were considered colonies when >50 cells were counted for neutrophils and monocyte-macrophages, >30 cells were counted for eosinophils, and when clusters consisted of >8 cells for CFU-E.

Variation. To assess the variation in number and type of colonies cultured from different bone marrow samples, aspirates were taken 1) from different calves, 2) from two different sternal sites per animal, and 3) from two animals at weekly intervals for a period of 5 weeks.

Staining. Erythroid colonies were not stained because they were easily identified by their red color. Megakaryocytes were picked from the MC medium and smears made which were rapidly dried using compressed air, fixed with absolute methanol, and stained with Giemsa.

Colonies grown in agar gels were stained in situ after 5 min fixation in citrate buffer (27 mM citrate, pH 3.6, 12 mM NaCl)/acetone/37% formaldehyde (25/65/8, vol/vol/vol). The fixed gels were washed with several changes of water. To detect eosinophils, the gels were then stained with Luxol fast blue (0.1% [wt/vol] in 70% [vol/vol]

Table 2. Growth factors (GF) and conditioned media (CM) tested in agar proliferation assays to support granulocyte-macrophage colony formation from bovine bone marrow progenitors

GF/CM tested	No. of colonies grown per 10 ⁵ mononuclear cells		
	0-50	50-250	250-650
IL2 (bovine recombinant)	×		
IL2 (human recombinant)	×		
IL3 (murine, WEHI 3B)	×		
GM-CSF (human recombinant)	×		
Post-endotoxin sera (bovine)		×	
CM ^a fetal liver (bovine)		×	
CM ^a fetal lung (bovine)		×	
CM ^a fetal spleen (bovine)		×	
CM ^a placenta (bovine)		×	
CM ^a PBL (bovine)		×	
CM ^a PBL (bovine, day 5 after Con A)			×

^a To produce CM, cells were stimulated with either Con A, PHA, or PWM, and supernatants tested separately at 1, 3, 5, and 7 days of incubation. PBL, peripheral blood leukocytes.

ethanol saturated with urea) for 30 min [15]. For neutrophils, staining was for 5 min with *p*-diaminodiphenyl benzidine (0.2% [wt/vol] free base in 40% [vol/vol] ethanol) and 0.003% (vol/vol) H₂O₂ [16]. The stained gels were washed over 30-50 min with repeated changes of water and counterstained for 2 min with hematoxylin (20% [vol/vol] in distilled H₂O). They were transferred into a dish of tap water where excess counterstain was removed, and, subsequently, floated onto 75- \times 40-mm slides, dried at 40°C, and examined at 400 \times magnification.

Results

Bovine bone marrow cells produced colony types and numbers comparable to those described for human and mouse (Table 1). The proportions of colony numbers and cell types grown from aspirates originating from either Boran or N'Dama breeds or from male or female calves did not differ significantly, and the results are therefore presented together.

After separation of the bone marrow cell suspension on Ficoll-Paque, the high-density cell pellet (>1.078 g/cm³) contained virtually all mature red blood cells and most (>95%) of the segmented granulocytes. The mononuclear cells found in this cell fraction were mainly late normoblasts, as determined by Giemsa-stained cytospin preparations. Proliferation assays revealed that few or no colonies were formed from this high-density cell population (up to 5 and 20 colonies per 10⁵ mononuclear cells, originating from GM-CFU and CFU-E, respectively). Between 1 and 5 \times 10⁷ nucleated cells per aspirate were recovered from the interface between Ficoll-Paque and medium, more than two-thirds of which belonged to the late erythroid lineage (basophilic to polychromatophilic normoblasts). The other most frequently identifiable cell types in this low-density cell fraction were immature neutrophil and eosinophil granulocytes (promyelocytes to bands), followed by erythroblasts, myeloblasts, lymphocytes, monocytes, and megakaryocytes. The proportions of nonnucleated cells obtained from the interface ranged from 40% to 70%. Approximately 90% of these cells were reticulocytes, as determined by Giemsa staining.

With respect to the number of granulocyte-macrophage colonies grown in agar assays, LCM collected on day 5 after

Table 3. Statistical evaluation of the numbers of colonies grown from different aspirates, per 10^5 mononuclear cells

	CFU-E	Granulocyte-macrophage	BFU-E
Sample size	24	35	22
Average	9.29 (767.1)	8.24 (315.3)	4.64 (48.8)
Geometric mean	9.21 (625.7)	8.23 (296.9)	0 (25.0)
SD	1.13 (427.9)	0.5 (119.5)	1.96 (52.6)
Minimum	5.2 (36)	7.28 (155)	0 (1)
Maximum	10.77 (1750)	9.53 (737)	7.39 (168)

The colonies were counted on day 5 (CFU-E), day 7 (granulocyte-macrophage), and day 10 (BFU-E). Due to the high variation, the original data (in brackets) were transformed to \log_2 in order to facilitate statistical analysis.

stimulation of peripheral blood leukocytes with Con A was superior to all other conditioned media and any growth factors tested (Table 2). LCM also supported formation of erythroid colonies (CFU-E and BFU-E) in MC medium and was, therefore, used in all subsequent progenitor assays. Conditioned media prepared from fetal bovine liver, lung, or spleen, as well as post-endotoxin sera, all supported some colony growth; however, none was as effective as LCM. Few or no colonies were formed when recombinant bovine or human IL2, murine IL3 (WEHI 3B), and hrGM-CSF were used as stimulating agents (Table 2).

Granulocyte-macrophage progenitors gave rise to a similar number, size, and type of colonies when grown in either MC or agar assays (Tables 1 and 3). The colonies appeared on day 4 and were counted on day 7. They began to disintegrate between days 8 and 14. The average number of granulocyte-macrophage colonies formed per 10^5 nucleated cells was 315 (Table 3).

Megakaryocytes grew best in MC medium. The cells had either developed to clusters of two to ten cells or to multinucleated single cells by days 10–12. There were fewer of this cell type (approximately 15 per 10^5 nucleated cells) compared to the other progenitors grown (Table 1).

Erythroid progenitors did not proliferate well in agar medium but, in MC, formed an average of 49 and 767 colonies per 10^5 nucleated cells originating from BFU-E and CFU-E, respectively (Tables 1 and 3). The proportion of CFU-E colonies grown in agar was between 1% and 10% compared to MC culture conditions, and no BFU-E were seen. In MC, CFU-E progenitors formed pink-to-red clusters or colonies of between eight and several hundred cells. Without prior staining, they were counted on day 5, and had lost their color by days 7 or 8. Colonies originating from BFU-E were counted on day 10 when they appeared as either single colonies (similar to larger CFU-E) comprised of between 20 and several hundred cells, or as a typical red "burst" formation consisting of 2–50 colonies (Fig. 1). When transferred to slides and stained with Giemsa, these colonies were found to consist of normoblasts and reticulocytes. They had usually disintegrated between days 12 and 15.

Microscopic evaluation of the entire agar disc after in situ staining with benzidine/hematoxylin showed dark blue nuclei and yellow-green cytoplasm in all granulocytes. Metamyelocytes, bands, segmenters, and disintegrating cells could easily be recognized. Eosinophil granulocytes of all devel-



Fig. 1. Portion of a large burst (BFU-E, day 11) grown in MC. It is comprised of single colonies (arrow), each of which consists of numerous individual cells (arrowhead). Due to the depth of the MC, not all colonies are in the focal plane (unstained, magnification $150\times$).

opmental stages (myelocyte to segmenter) appeared bright turquoise-blue after staining with Luxol fast blue. The cell size of mature eosinophils appeared smaller than that of neutrophils, and the number of cells per colony (30–1000) was usually lower compared to that of neutrophils, which ranged from several hundred to thousands. There were two types of colonies that contained cells with the morphological appearance of monocyte-macrophages, one being compact and the other showing uniformly dispersed cells. Cells from both of the latter colony types were negative to benzidine staining. The cell nuclei in the compact colony type were either round or kidney shaped with a nucleus-to-cytoplasm ratio greater than 1:2, whereas the round nuclei of the dispersed cells were significantly smaller. The cell numbers, per colony, were comparable with those of the neutrophils.

Granulocyte-macrophage colonies evaluated on day 7 exhibited three major shapes, with similar distributions in both culture systems. The proportions for compact, dispersed, or intermediate (compact center and spread periphery) colony shapes were approximately 40%–60%, 30%–40%, and 10%–20%, respectively. The majority of the compact colonies was comprised of benzidine-negative cells and had large nuclei. Most of the intermediate colonies contained benzidine-positive granulocytes, whereas the dispersed colonies consisted mainly of unstained monocyte-macrophages. Eosinophil colonies could, with some experience, be distinguished in unstained gels from neutrophil or monocyte colonies by the smaller size of both the colony and the individual cells, whereas the colony shape, in most cases, was of the intermediate type. Examination of colony morphology in unstained gels, however, can only give a preliminary determination of the cell type formed, and an exact evaluation requires specific stains.

The variation in the numbers and types of colonies formed from different bone marrow biopsies was very great. It made no difference whether the aspirates were taken from different sternal sites (Fig. 2) or at various times from the same animals (Fig. 3), or whether the bone marrow cells were aspirated from different calves (Fig. 2). Colony counts, per 10^5 mono-

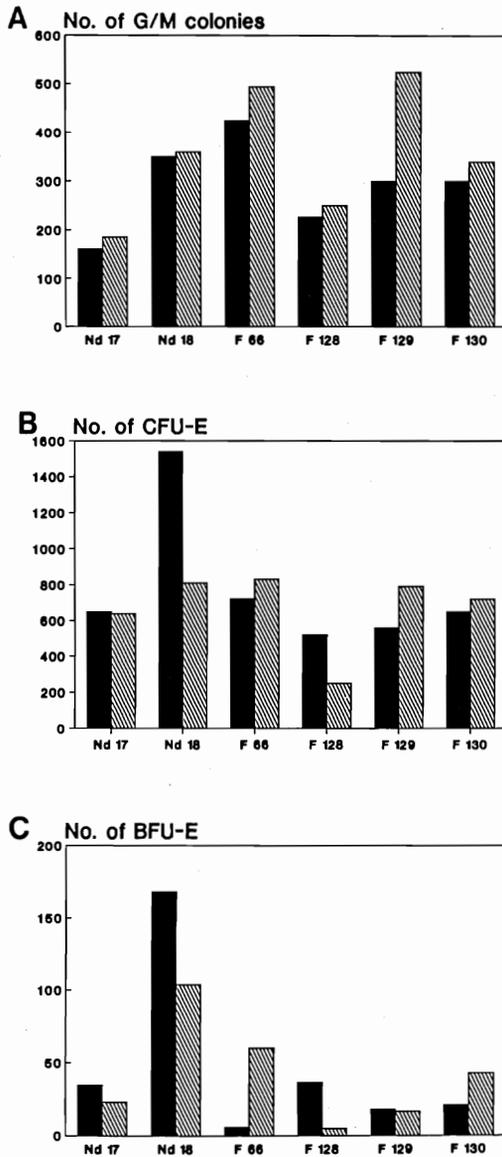


Fig. 2. Number of colonies formed, per 10^5 bovine mononuclear bone marrow cells inoculated. (A) granulocyte-macrophage; (B) late erythroid progenitors (CFU-E); and (C) early erythroid (BFU-E) progenitors. The figure shows aspirates from six cattle, with two sternal sites per animal (*hatched and solid bars*). The values shown are representative for > 50 cattle tested. Animal identification numbers are given below the *abscissa*.

nuclear cells, varied from 1 to 168 for BFU-E, from 36 to 1750 for CFU-E, and from 155 to 737 for the granulocyte-macrophage lineage (Table 3). Due to the high variation observed, \log_2 transformation was carried out on the raw data in order to facilitate statistical analysis (Table 3). Evaluation of the granulocyte-macrophage cell types also exhibited a high degree of variation (Fig. 4); the arithmetic means (\pm sample SD) from 12 examinations were 44.2% (\pm 9.3) for monocyte-macrophages, 27.8% (\pm 6.9) for neutrophils, 16.2% (\pm 5.9) for eosinophils, and 11.8% (\pm 5.5) for mixed granulocyte-macrophage colonies.

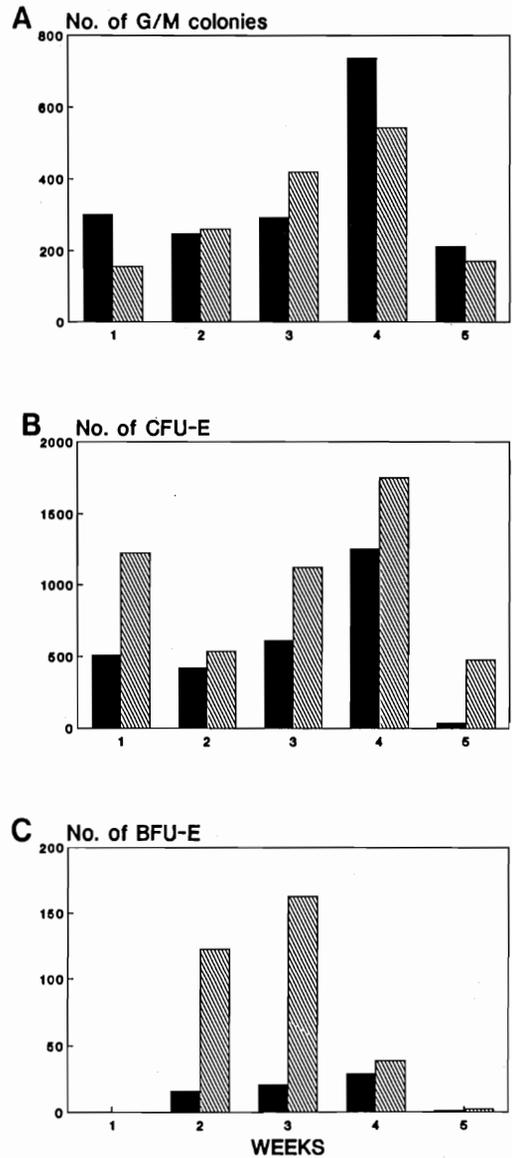


Fig. 3. Numbers of colonies formed from bovine granulocyte-macrophage (A), late erythroid (CFU-E) (B), and early erythroid (BFU-E) (C) progenitors. The marrow aspirates were taken from two Boran calves (*hatched and solid bars*) at weekly intervals.

Discussion

This paper reports the formation of myeloid colonies from bovine bone marrow progenitors in semisolid media. The colonies originated from single cells, as confirmed by the linear relationship between the numbers of cells inoculated and colonies formed; this is in accordance with earlier reports for the mouse [1] and the cow [9].

Although comparable numbers of granulocyte and macrophage colonies were grown in both agar and MC-based culture media, agar was superior for these cell types. Apart from the fact that it is easier to handle, microscopic examination after *in situ* staining of the entire agar disk allowed a more exact evaluation of the cell types present, because the original morphology of the colonies was retained. MC-based

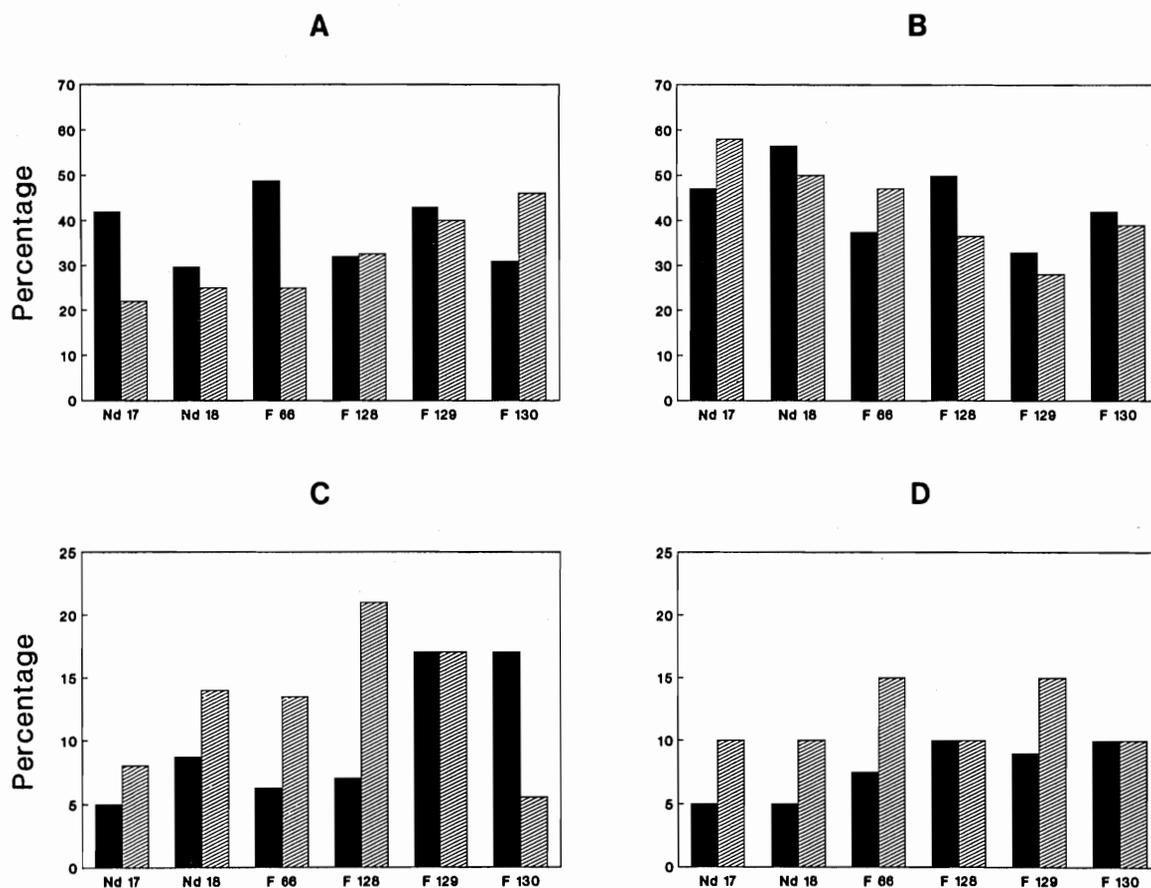


Fig. 4. Cell types (as percent of granulocyte-macrophage colonies) of day-7 myeloid colonies grown from bone marrow aspirates from six calves, from two sternal sites in each (*solid and hatched*). The values shown for the 6 animals were similar in all tests and are representative of 20 calves examined. Animal identification numbers are given below the *abscissa*. (A) neutrophil granulocytes; (B) monocyte-macrophages; (C) mixed granulocyte-macrophage colonies; and (D) eosinophil granulocytes.

medium, on the other hand, remained quite soft over the incubation period, and the colonies tended to lose their typical shapes; they appeared more diffuse and were sometimes found at the bottom of the culture dish. For microscopic examination of cells grown in MC, the colonies had to be picked off and transferred onto slides [7], a procedure that may lead to misinterpretation, because contamination with cells from neighboring colonies cannot be ruled out. Consequently, the number of mixed colonies determined would be higher than the actual number present.

Recent reports have described different staining techniques for hemopoietic colonies grown in agar. Bol et al. [17] used a tetrazolium salt that stained all colonies red. Although this method simplifies and accelerates the screening procedure, it does not differentiate between the various cell types. The esterase stains described by Kubota et al. [18] and Phillips et al. [19] proved unsatisfactory in our hands for bovine cells grown in agar, because little or no naphthol AS-D chloracetate esterase was observed in granulocytes. The alpha-naphthyl acetate esterase stain (ANAE) proved to be unreliable, because some cells with the morphology of monocytes stained intensely, whereas others showed only a few stained foci or did not stain at all. Conversely, some cells that presented the morphological appearance of granulocytes gave a positive

reaction for ANAE. In contrast, benzidine-hematoxylin produced minimal background staining in the gels, and neutrophils could easily be distinguished from monocytes by their yellow-green cytoplasm. Therefore, the benzidine-negative cells in most mixed granulocyte-macrophage colonies appear to be monocytes with large nuclei and correspond to those cells that stained less intensely for ANAE. It should be borne in mind, however, that these cells may also be earlier developmental stages of granulocytes (for example, myeloblasts or myelocytes), and further investigation is necessary in order to identify the different immature cells in culture.

Cell aggregates of bovine eosinophils, the identification of which we report herein for the first time, were often found to produce clusters of only 5–20 cells. Larger colonies formed also consisted of fewer cells than those of neutrophils and monocytes. This may indicate proliferation of a later developmental stage of progenitors seeded or a lack of supporting growth activity of the conditioned medium used.

Erythroid progenitors proliferated well in MC, and colonies were formed from both the early (BFU-E) and the late (CFU-E) progenitors. Although bovine CFU-E have been successfully cultured in plasma clots [7] and MC [10], formation of bovine BFU-E has not previously been reported. Whereas colonies originating from CFU-E had disappeared

by days 7 or 8, the colonies formed from BFU-E were a bright, pink-red color at days 9–11. They were comprised of either one or a few cell aggregates (comparable to larger CFU-E) or they had a higher number of cell clusters (5–50), exhibiting the typical “burst” formation (Fig. 1). This variation in size suggests different stages of maturation of the respective progenitors, and the small and large colonies may indicate later or earlier BFU-E, respectively, as reported for human [20] and mouse [21] bone marrow cultures.

Kaaya et al. [11] described day-5 colonies derived from CFU-E, 1%–5% of which contained megakaryocytes. We did not observe this type of mixed colony. It seems unlikely that cells with the capacity to give rise to both megakaryocytes and erythrocytes could form colonies within 5 days, whereas the earlier erythroid progenitors (BFU-E), which produce only red blood cells, require 10 days. Furthermore, megakaryocyte progenitors required 10–12 days to develop to either clusters of two to ten cells or to multinucleated single cells corresponding to the final cell stage IV described by Levine et al. [22]. In these studies [11] it is possible that bone or tissue pieces that contained both CFU-E and megakaryocyte progenitors had not been removed prior to cultivation. Our own observations showed that, after separating bone marrow suspensions on Ficoll–Paque, the interface contained various amounts of these natural cell aggregates. In subsequent culture they often formed “mixed colonies,” which were not found if the interface cells had been filtered through cotton gauze. In the latter case, we observed mixed colonies comprised of only granulocytes and macrophages.

Although the culture conditions were the same, we observed a high variation in the number and the type of granulocyte–macrophage and erythroid colonies grown from different aspirates. This is in agreement with other reports on bovine bone marrow progenitors [9, 11]. A major reason for such variability may be the source from which bone marrow is aspirated in the cow, where the sternum was found most suitable [12]. In other species this is less of a problem. In the mouse, for example, the entire marrow content of the femur is collected and, in man, bone marrow is normally aspirated from the iliac crest [20], which provides larger amounts of a more fluid marrow. The state of hemopoiesis of individual animals did not seem to be the reason for such variation, because we found variability in colony number and type with marrow taken from different sternal sites within the same animal. It seems likely that the position of the needle when inserted into the sternum could lead to variability in the types of progenitor cells aspirated.

The studies described in this communication represent the initial phase of investigations that ultimately aim at the identification of a possible connection between pancytopenia and the pathological sequelae of African trypanosomiasis. Such studies would have to take into account variability between individual animals, whether infected or not. Pooled marrow aspirates from several animals could reduce such variation. Monoclonal antibodies, either specific for different progenitor cell populations or for unwanted cell types, used in combination with fluorocytometry to purify early bone marrow cells, might provide a useful tool to circumvent this problem. We are currently investigating this approach.

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