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Normal Range of Blood Colony-forming Cells (CFU-C) in Humans: Influence of Experimental Conditions, Age, Sex, and Diurnal Variations*

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Schwankungsbreite von koloniebildenden Zellen (CFU-C) im Blut des Menschen: Einfluß von experimentellen Bedingungen, Alter, Geschlecht und tageszeitbedingten Schwankungen

Zusammenfassung: Koloniebildende Zellen (CFU-C) aus dem Blut und Koloniestimulierte Aktivität aus dem „feeder layer" peripherer Blutleukozyten (Leukozyten-CSA) wurden in einem halbflüssigen Agarkultur-System bei 69 Normalpersonen untersucht. Gesunde Freiwillige wurden nach Geschlecht und Alter (20–45 Jahre und älter als 60 Jahre) in Gruppen unterteilt. Der Anteil der zirkulierenden CFU-C war bei jungen Frauen (20–45 Jahre) signifikant niedriger als in der Gruppe älterer Männer (älter als 60 Jahre), jedoch waren die Unterschiede zwischen den übrigen Gruppen nicht signifikant. Leukozytäre CSA unterschied sich in den verschiedenen Gruppen nicht signifikant. Bei 5 jungen Männern wurden zirkulierende CFU-C am selben Tag morgens um 8.00 und nachmittags um 16.00 Uhr untersucht: Es ergaben sich keine unterschiedlichen Werte. Bei 18 Personen wurden die Untersuchungen nach längeren Zeitintervallen wiederholt: Die Anzahl der gebildeten Kolonien variierte maximal um das Fünffache. Plasma und segmentkernige Granulozyten, wie sie im angewandten Kultursystem üblich waren, inhibierten das Wachstum der Kolonien nicht. In den meisten Fällen ließen sich in doppelschichtigen Kultursystemen höhere Koloniezahlen erreichen als in einschichtigen, jedoch schienen die „feeder layer" einiger normaler Personen das Koloniewachstum zu inhibieren.

Schlüsselwörter: CFU-C – Leukozyten CSA – tageszeitbedingte Schwankungen

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Summary. Blood colony-forming cells (CFU-C) and colony-stimulating activity obtained from feeder layers of peripheral blood leucocytes (leucocyte CSA) have been studied in 69 normal subjects by means of semisolid agar culture system. Groups of normal volunteers were selected according to sex and age (20 to 45 and older than 60 years) and the results compared. The mean number of circulating CFU-C was significantly lower in young women (20–45 years old) than in males over 60 years of age, but no differences were found among the other age and sex groups. Leucocyte CSA did not significantly differ among these groups. In 5 young males the blood CFU-C did not show significant variations at 8 AM and at 4 PM of the same day. When the study was repeated in 18 subjects at longer time intervals, the number of colonies showed a maximum fivefold variation. The amount of plasma and polymorphonuclear granulocytes present in our culture system did not inhibit the colony growth. In most cases, double layer cultures grow a higher number of colonies than single layer, but feeder layers of some normal subject seem to inhibit the colony growth.

Key words: CFU-C – Leucocyte CSA – Diurnal and ultradial variations

The differences in the mean values and the wide ranges of the blood CFU-C of normal humans reported by different laboratories [1–12, 14, 16–19] are due in part to the different methods employed, such as single or double layer cultures, use of leucocyte feeder layer or conditioned medium, different amount of leucocytes in culture, use of unwashed white blood cells or of separated mononuclear cells, and the minimum number of cells scored as colony. Moreover, the influence of different physiological conditions on blood CFU-C and on CSA obtained from feeder layers of peripheral blood leucocytes (leucocyte CSA) has not yet been fully investigated in man, so that uncertainty may arise when these cells are studied in pathological conditions.

Present experiments were undertaken to determine if age, sex, diurnal variations, or feeder layer of different subjects can account at least in part for the wide range of blood CFU-C observed in normal humans. Moreover, the influence of plasma and polymorphonuclear granulocytes present in culture and of single or double layer culture on the colony growth has been studied.

Materials and Methods

Groups of Subjects

Sixty-nine haematologically normal volunteers including 37 men and 15 women aged from 20 to 45 years and 8 men and 9 women over 60 years of age have been studied.

Culture Methods

CFU-C were obtained from 10 ml of peripheral blood and a feeder layer of blood white cells was used as a source of CSA. The culture method was that described by Kurnick and Robinson [9] modified by adding methylcellulose 1 : 20 to the blood sample to increase the sedimentation rate of the erythrocytes. The culture medium was a modified preparation of the McCoy 5A medium with 15% fetal calf serum. One milliliter of medium with 0.5% purified agar (Difco) and 10^6 peripheral leucocytes was placed into 35 mm Falcon Petri dishes as feeder layer. Dishes were

incubated at 37° C for 24 h before adding an overlayer consisting of 1 ml of medium, 0.3% purified agar, and 10^6 peripheral leucocytes as a source of CFU-C.

Peripheral blood leucocytes from 20 to 45 years old normal males were used for all different groups of subjects, both as feeder layers to study the CFU-C growth and as source of target cells to evaluate the leucocyte CSA.

In 55 subjects both double and single layer cultures were set up.

Colonies were counted with an inverted microscope after 12 days of incubation at 37° C in a highly humidified atmosphere of 7.5% CO_2 in air. Triplicate cultures were set for each subject. Only aggregates of more than 50 cells were scored as colonies and expressed for 1×10^6 cells.

Influence of Autologous Plasma Present in Culture

Cultures prepared as previously described were compared with cultures of washed cells reintegrated with autologous plasma or McCoy 5A medium. Further, CFU-C growth was compared in cultures with washed cells of the same subject supplemented with increasing doses of autologous plasma (0.5, 0.75, 1.0, 1.25 ml).

Influence of Polymorphonuclear Granulocytes Present in Culture

In 5 cases double layer cultures of 1×10^6 unfractioned white blood cells were compared with double layer cultures of 1×10^6 mononuclear cells obtained with a Ficoll-Hypaque gradient separation method [16]. In 1 case 1×10^6 mononuclear cells were cultivated on feeder layers containing 1×10^6 mononuclear cells alone, or 1×10^6 mononuclear cells plus 0.5×10^6 and 1×10^6 polymorphonuclear granulocytes. Further, in the 69 normal subjects the number of colonies grown per 10^6 unfractioned white blood cells was compared to the number of polymorphonuclear granulocytes present in both over and feeder layers, ranging from 0.82×10^6 to 1.65×10^6.

Statistical Analysis

Confidence limit factors at level of 95% of the mean (L.F.) were calculated and comparisons were made with Student's *t*-test.

Results

Blood CFU-C in the Whole Group of Normal Subjects

In 69 normal subjects the study showed a mean colony growth of 31.95 ± 1.15 colonies/10^6 leucocytes (L.F. 29.32–33.88), the colony numbers ranging from 5.75 to 74.75.

Blood CFU-C and Leucocyte CSA in Subjects of Different Age and Sex

Blood CFU-C were 33.15 ± 1.48 (L.F. 30.19–36.11) in 37 males aged 20–45 years, 27.64 ± 2.40 (L.F. 22.84–32.44) in 15 females aged 20–45 years, 33.97 ± 0.35 (L.F. 33.27–34.67) in 8 males over 60 years, and 36.82 ± 4.26 (L.F. 28.30–45.34) in 9 females over 60 years.

Leucocyte CSA was 33.15 ± 1.48 (L.F. 30.19–36.11) in 37 males aged 20–45 years, 32.12 ± 0.72 (L.F. 30.68–33.56) in 15 females aged 20–45 years, 34.53 ± 1.32 (L.F. 31.89–37.17) in 8 males over 60 years, and 34.71 ± 1.27 (L.F. 32.17–37.25) in 9 females over 60 years.

Significant differences were observed only between women aging 20–45 and men over 60, CFU-C values being lower in the young women. Leucocyte CSA did not differ significantly among the different groups.

Table 1. Colony growth by 1×10^6 peripheral blood leucocytes (PBL) in 18 males aging 20 to 44 years studied at different time intervals. In patients 1–16 up to 8 investigations were performed during a period of 1 month

Subjects	Time interval between first and last culture	Number of colonies/l $\times 10^6$ PBL							
1	1 month	61.0	62.50						
2	1 month	10.33	15.75						
3	1 month	24.66	26.00						
4	1 month	43.50	46.25						
5	1 month	13.67	22.66						
6	1 month	14.75	50.65						
7	1 month	41.75	65.75						
8	1 month	30.50	34.25	52.75					
9	1 month	32.50	40.00	48.25					
10	1 month	49.50	57.00	57.75					
11	1 month	24.33	29.50	40.25					
12	1 month	31.33	50.00	67.66					
13	1 month	31.00	31.75	35.25					
14	1 month	38.00	41.00	44.50	48.50				
15	1 month	10.00	18.00	25.00	27.50	29.50			
16	1 month	9.66	15.00	18.50	21.00	27.75	31.50	38.75	56.00
17	3 months	42.00	55.00	74.50					
18	7 months	7.75	24.50						

Diurnal Variations of Blood CFU-C

In 5 males aged 21 to 24 years, cultures were set up at 8 AM and at 4 PM. Blood CFU-C showed a mean diurnal variation of 19%, ranging from 3 to 39%.

Blood CFU-C in the Same Subject Studied at Long Time Intervals

Eighteen males aged 20 to 44 years were studied at different time intervals, using for each subject always the same source of CSA (Table 1). The intervals of time were of 1 month in 16 cases, of 3 months in 1 case, and of 7 months in a further case. The number of CFU-C in each individual case showed a maximum fivefold variation, being lower than twofold in 13 subjects (72% of cases), between twofold and threefold in 3 subjects (17% of cases), and higher than threefold in 2 subjects (11% of cases).

Variation of the Colony Growth Using Feeder Layers from Different Subjects

The results obtained from culturing on the same occasion overlayers from each of 4 different subjects on feeder layers from 8 different volunteers are reported in Table 2. The number of colonies from each subject showed a maximum variation of 5.6 times. The feeders from only 3 subjects (numbers 2, 4, 7) determined a consistant increase of the colony growth, while inconsistant results were obtained with feeders from the other 5 subjects.

Table 2. Colony growth by 1×10^6 peripheral blood leucocytes (PBL) of four different normal subjects plated over PBL feeder layers from eight different normal subjects

Over-layers	No feeder (single strate culture)	Number of colonies/1×10^6 PBL grown on: Feeder 1	Feeder 2	Feeder 3	Feeder 4	Feeder 5	Feeder 6	Feeder 7	Feeder 8
A	16.25	10.00	31.33	31.66	25.00	13.00	8.60	27.66	14.00
B	8.00	14.66	29.50	16.75	27.50	12.75	12.50	16.75	21.66
C	6.50	15.00	24.25	7.50	32.75	6.25	12.50	16.25	19.33
D	10.00	10.75	59.75	27.75	38.50	10.00	16.00	19.00	40.33

Comparison Between Single and Double Layer Cultures

In 55 subjects mean colony growths of $20.74 \pm 1.62/10^6$ leucocytes (L.F. 23.98–17.50) and of $25.13 \pm 1.75/10^6$ leucocytes (L.F. 28.63–21.63) were obtained in single and double layer cultures respectively. The difference between these two mean values is not statistically expressive and a correlation "r" value of 0.59 is obtained, corresponding to r^2 of 0.35, viz., only 35% ("r" significance at level of $p < 0.001$).

Influence of the Amounts of Autologous Plasma Present in Culture on the Colony Growth

Cultures of washed cells resuspended in McCoy 5A medium or in 0.5 to 1.25 ml of autologous plasma grew the same number of colonies as cultures of unwashed cells.

Influence of Polymorphonuclear Granulocytes in our Culture System

In the 69 normal subjects no correlation was found between the number of granulocytes present in culture and the number of colonies grown in each individual case.

In 5 cases the culture of mononuclear cells alone gave a number of colonies ranging from 0 to 8, with a mean value of 3.64 which was significantly lower than that obtained when whole blood leucocytes were cultivated. In 1 case when 1×10^6 mononuclear cells in the overlayer were cultivated on feeder layers containing mononuclear cells alone, or mononuclear cells plus 0.5×10^6 or 1×10^6 polymorphonuclear granulocytes, the number of colonies obtained rose from 3.5 to 14.5 and further to 26.5.

Discussion

In 69 normal subjects we observed mean values of blood CFU-C higher than those reported by other authors using the same culture method [1,4,5,7,9,19], while the colony range was as great as the one they had observed.

Our results do not show an inhibitory effect of polymorphonuclear granulocytes and plasma on the colony growth, at least within the amounts present in our culture system. A feeder layer of 1×10^6 peripheral blood leucocytes is as-

sumed to provide an amount of stimulus in excess of that needed for a maximum colony growth from bone marrow [13]. Although CSA-producing cells are present in both feeder and overlayers of blood leucocytes, single layer cultures seem to grow a lower number of colonies than double layer cultures [5]. Present experiments confirm a lower mean number of colonies in single layer cultures, but the difference with the number of colonies obtained in double layer cultures is not statistically significant. However, the large variability in individual cases and the correlation of only 35% between single and double layer cultures make it doubtful that the two experimental conditions are comparable. The results obtained by stimulating the overlayer of the same subject with feeder layers obtained from different subjects are compatible with the presence in the feeder layer of some normal subjects of colony growth inhibiting factors. The wide colony range observed by us and by others may be due in part to these factors, as well as to the different number of circulating CFU-C in normal subjects.

When the influence of sex and age on the colony growth was examined significant differences were observed only between women aging 20 to 45, and men older than 60, CFU-C mean values being significantly lower in the young women. Other workers comparing males and females independently from age found a significantly lower number of colonies in bone marrow [15] and blood [1] from females. The differences due to age and sex alone do not account for the different mean colony growth reported by various laboratories but they must be kept in consideration when CFU-C are studied in pathological conditions. This is not of particular relevance for the study of systemic haemopathies presenting large modifications of the blood CFU-C, but it is important for the study of those conditions presenting only minor changes of these cells.

The non-significant diurnal variations in the number of colonies compared to the greater than twofold variations observed in 28% of subjects studied at longer time intervals seem to indicate that in physiological conditions the number of blood CFU-C is not constant, but that it changes because of biological factors not yet understood. Further studies allowing a thorough statistical analysis for the identification of possible cyclic oscillations are in progress.

Since the normal range of blood CFU-C is so large, it is necessary to study numerous examples of any given morbid condition of to be able to demonstrate the significance any quantitative alteration.

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