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THE ENHANCED COLONY GROWTH OF MOUSE CFU-c BY HUMAN SERUM

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The addition of human serum to colony stimulating factor (CSF) enhances the growth of granulocyte and macrophage colonies derived from C_{57} BL/6 mouse marrow cells. The growth-enhancing effect of normal human serum was dose-dependent and observed in various colony assay systems employing conditioned medium prepared from L-cells, human peripheral blood leukocytes, C^{57} BL/6 mouse whole embryo and sera from C^{57} BL/6 mice injected with E. coil endotoxin. The enhancing activity was also shown in sera of mice, rats and rabbits but not in those of guinea pigs and fetal calves. The enhancing activity of sera from patients with various hematological diseases, so far assayed, does not significantly differ from that of normal control sera. The active principle in human sera was heat-stable, non-dialysable, not extractable by ether and separated in the lower molecular weight zone corresponding to albumin and Ig G by gel filtration of Sephadex G-200. Neither pure human albumin nor Ig G showed any enhancing effect on the colony formation. On the other hand, gel filtration of normal human serum on Sephadex G-200 demonstrated the inhibitory component of the colony growth in the higher molecular weight zone corresponding to Ig M. As the pretreatment of marrow cells with the active principle in human sera did not show any effect on the colony growth and the coexistence of the active principle in human sera and CSF was required for the enhanced colony growth, the cooperative action of the active principle in human sera and CSF is thought to be required for the enhancement of the colony growth.

INTRODUCTION

The progenitors of granulocytes and macrophages, designated as colony forming unit in culture (CFU-c), can proliferate and form colonies in semi-solid agar medium containing an adequate concentration of a specific glycoprotein, i.e., colony stimulating factor (CSF).[1,2] Human marrow cells can form colonies and clusters of CFU-c without exogenous CSF, while the addition of exogenous CSF to agar cultures is essential for the colony growth of mouse granulocyte and macrophage progenitors. Exogenous CSFs are usually supplied from feeder layers of peripheral leukocytes, marrow or spleen cells or media conditioned by leukocytes or other tissue cells.[3~5] In addition to feeder layers or conditioned media, an aliquot of fetal calf serum is usually added to culture media in order to obtain an appropriate colony growth. Moreover, it has been shown that when a small amount of human serum is added to marrow cultures stimulated by exogenous CSF, the colony growth increases in a super-additive way.[6~9] This super-additive effect of human serum on the colony growth stimulated by CSF requires the precise analysis on its nature, as clear understanding on this mechanism is still to come.

The present experiments were undertaken to delineate the nature of this enhancing effect of human serum on the colony growth of granulocyte and macrophage progenitors in

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semisolid agar cultures of mouse bone marrow cells.

MATERIALS AND METHODS

Assay of the colony stimulating activity

The colony stimulating activity (CSA) of various sources was assayed by the double-layer agar culture technique.[10] Bone marrow cells to be cultured were obtained from the femora of 2 to 3 month old female C_{57} BL/6 mice. Bone marrow cells were collected in McCoy 5A medium, then dispersed by gentle pippeting with a syringe. Non-adherent marrow cells were separated from adherent cells by the method of Messner *et al.*[11] Upper-layers were prepared by mixing 0.9 ml of McCoy 5A medium with 15% fetal calf serum (GIBCO) including 2×10^5 marrow cells and 0.1 ml of boiled 3% agar (DIFCO, Bacto-agar). Under-layers were prepared by mixing 0.7 ml of McCoy 5A medium, 0.1 ml of L-cell conditioned medium, 0.1 ml of test serum and 0.1 ml of boiled 5% agar. As a control, 0.1 ml of phosphate buffered saline was put into under-layers instead of the test serum. When the dose-response effect of enhancing capacity of the human serum on the in vitro colony formation was assayed, aliquots of the test sera were added into under-layers containing 0.3 ml of McCoy 5A medium, 0.1 ml of L-cell conditioned medium and 0.1 ml of boiled 5% agar, and final volume of under-layers was adjusted to 1.0 ml by adding phosphate buffered saline. After 10 days culture in a fully humidified atmosphere of 7.5% CO_2 in air, colony number was counted by an inverted microscope (Nikon Co.), scoring all aggregates of 50 or more cells as colonies. As an endogenous colony formation was observed in some cultures containing 0.1 ml of test serum and 0.1 ml of phosphate buffered saline instead of L-cell conditioned medium, net colony numbers in cultures containing both test serum and L-cell conditioned medium were obtained by the subtraction of endogenous colony numbers in cultures described above from those in cultures containing both test serum and L-cell conditioned medium.

The Student's t-test was used to evaluate the differences between the number of colonies in the control cultures and that in the test sample containing cultures. The significant difference from the control was evaluated as positive at $P < 0.05$.

Blood samples were collected from rabbits, rats, mice (C_{57} BL/6), guinea pigs, fetal calves, normal human donors and patients with hematological disorders. The blood was allowed to clot at room temperature for 3-5 hours and then centrifuged at 2,000 g for 15 minutes. The serum was removed and stored at $-20°C$ until use.

Conditioned media used as CSF in the present study were harvested from 4 days cultures of L-cells,[12] 4 days cultures of 18-day C_{57} BL/6 mouse whole embryo cells in minimum essential medium containing 15% fetal calf serum[13] and 7 days cultures of human peripheral leukocytes in McCoy 5A medium containing 15% fetal calf serum.[14] Pooled sera of C_{57} BL/6 mice injected i.p. 5 hours previously with 5 μg E. coli endotoxin (DIFCO)[15] were also used as CSF.

Analysis of physico-chemical properties of the serum colony enhancing factor

In order to analyse physico-chemical properties of the serum colony enhancing factor, dialysis, heat treatment, ether treatment and fractionation of sera were performed.

Dialysis of sera; Human sera were dialysed in 1/4 inch dialysis tubing for 72 hours at 4°C against distilled water. Then, the euglobulin precipitate and the supernate were separated

by centrifugation. The precipitate was redissolved in the original volume of phosphate buffered saline and its capacity to enhance the colony growth was studied.
Heating of sera; Human sera were heated for 30 minutes in a water bath at 50°C, 56°C, 60°C and 65°C respectively.
Ether treatment of sera; Equal volumes of sera and ether were mixed by a vortex mixer and incubated at 4°C for 6 hours with occasional shakings. The lower aqueous layer was removed by pippetes and analyzed for its enhancing activity on the colony growth.
Fractionation of serum on gel filtration; Two and a half ml of human serum was placed on a Sephadex G-200 column, 2.6 × 45 cm, running at a flow rate of 0.3 ml/min. at 4°C. The buffer used was 0.01 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. Each fraction consisting of 3 ml was dialyzed overnight against water. Protein concentration was measured by optical density at 280 μm. Two to eight of fractions were regarded as one group, then the volume of each group was concentrated to 1/3 of the original by collosion bags (MS Co.), 0.45 μm-millipore filtered and analysed for its effect on the colony.

Human albumin and Ig G purchased from commercial sources were assayed for their effect on the colony growth. The human albumin (SIGMA Co.) or human Ig G (Miles Co.) was dissolved in 0.01 M Tris-HCl buffer pH 7.4, containing 0.15 M NaCl at various concentrations and 0.1 ml of each solution was added to the under-layers of cultures.

Protein concentration of samples was measured by the Lowry's modification of the Folin method.[16)]

Analysis of the interaction between the serum colony enhancing factor and CSF

In order to analyse the interaction between the serum colony enhancing factor and CSF, two-step cultures were performed. Bone marrow cells of C_{57}BL/6 mice at the concentration of 5×10^7/ml were preincubated with the medium containing 1.6 ml of McCoy 5A medium and 0.4 ml of the serum colony enhancing component for 24 hours at 37°C. Then, after washing with phosphate buffered saline, 2×10^5 cells were used for the assay of CFU-c.

RESULTS

Enhancing activity of normal human serum

Figure 1 shows the enhancing effect of normal human serum on the in vitro colony growth of C_{57} BL/6 mice marrow cells in the presence of L-cell conditioned medium. A proportional dose-response relationship was observed up to 0.15 ml of serum added, at which dose the maximal enhanced colony growth was obtained, being enhanced by 7 times of the standard. The addition of normal human serum also increased the size of individual colonies but did not stimulate any preferential growth of granulocyte or macrophage colonies.

The colony number from 2×10^5/ml of non-separated marrow cells stimulated by human serum and L-cell conditioned medium was 86 ± 24 (Mean ± S.D)/plate, while that of 2×10^5/ml of non-adherent cells was 73 ± 31/plate.

The degree of the enhancing effect of normal human serum was compared in the colony formation stimulated by CSFs from various origins. As shown in Table 1, the enhancement of the colony growth was observed in cultures containing conditioned medium prepared from human peripheral leukocytes, conditioned medium prepared from C_{57} BL/6 mouse

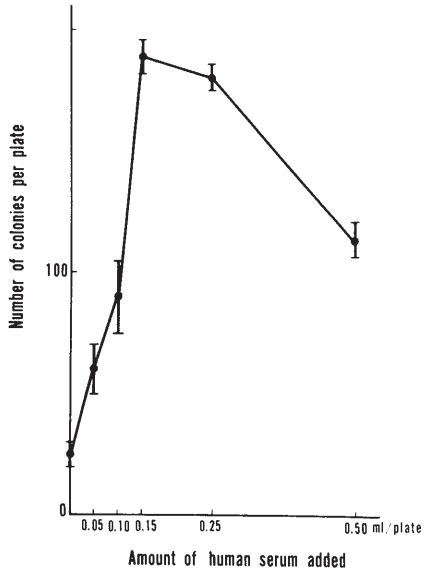

Fig. 1

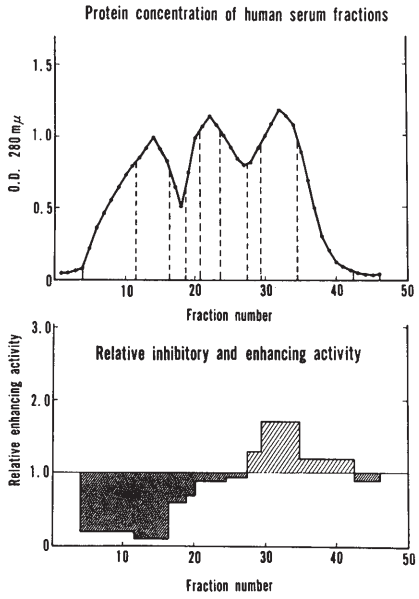

Fig. 2

Fig. 1. The enhancing effect of the addition of human sera on the in vitro colony formation in the presence of L-cell conditioned medium.
One tenth ml of L-cell conditioned medium and various amounts of normal human sera were added to the under-layers. Mean colony counts and standard deviations from triplicate cultures are given.

Fig. 2. The enhancing and inhibitory activity of fractions of human sera fractionated by Sephadex G-200 column chromatography.
Two to eight fractions, as indicated by broken lines, were collected in groups, concentrated to 1/3 of the original by collosion bags, 0.45 μm-millipore filtered and analyzed for its effect on the colony growth. The term of 'Relative enhancing activity' represents the ratio between the mean colony number of the control culture and that of the test sample containing culture.

Table 1. The effect of the addition of human sera on the in vitro colony formation in the presence of various CSFs.

Source of CSF	Number of colonies per plate CSF + phosphate buffered saline	CSF + normal human serum	Relative enhancing activity	P value
L-cell conditioned medium	83 ± 17	383 ± 31	4.6	$P < 0.05$
Human peripheral leukocytes conditioned medium	54 ± 16	106 ± 19	2.0	$P < 0.05$
C_{57} BL/6 mouse whole embryo conditioned medium	94 ± 23	176 ± 39	1.9	$P < 0.05$
Sera of E. coli endotoxin injected C_{57} BL mice	128 ± 29	269 ± 35	2.1	$P < 0.05$

whole embryo and sera from C_{57} BL/mice injected with E. coli endtoxin as well as conditioned medium prepared from L-cells.

Enhancing activity of sera from animals of various species

Table 2 shows the effect of the sera from various animals on the colony growth. The sera from rabbits, rats and C_{57} BL/6 mice were found to have the capacity to enhance the colony growth. Human sera exhibited the highest enhancing activity, while fetal calf serum did not show any enhancing effect nor inhibitory effect. Sera from guinea pigs seem to exhibit the inhibitory effect.

Enhancing activity of the sera from patients with various hematological diseases

Table 3 shows the results of an experiment to assess the enhancing activity of the sera from patients with aplastic anemia, polycythemia vera and myelogenous leukemia. No overall difference on the degree of the enhancing activity was observed among sera from patients with various hematological diseases.

Physico-chemical properties of the colony enhancing factor in human serum

Table 4 shows the summarized results on the physico-chemical properties of the colony enhancing factor in the human serum. The dialyzed sera showed full capacity to enhance the colony growth but the redissolved precipitate showed no enhancing activity. The serum heated up to 60°C for 30 minutes did not enhance the colony growth. Ether treatment of serum did not affect its enhancing activity.

When human serum was fractionated by gel filtration using Sephadex G-200 column, the enhancing activity was localized in the lower molecular weight zone corresponding to albumin and Ig G as shown in Figure 2. Neither pure human albumin nor Ig G from commercial sources showed any enhancing effect on the colony formation as shown in Table 5.

Table 2. The effect of the addition of sera from various animals on the in vitro colony formation in the presence of L-cell conditioned medium.

Source of serum		Number of colonies per plate	Relative enhancing activity	P value
Human	no. 1	546 ± 26	4.6	$P < 0.05$
	no. 2	627 ± 25	4.6	$P < 0.05$
	no. 3	568 ± 23	4.1	$P < 0.05$
Rabbit	no. 1	253 ± 24	1.8	$P < 0.05$
	no. 2	249 ± 27	1.8	$P < 0.05$
	no. 3	243 ± 16	1.8	$P < 0.05$
Mouse (C_{57} BL/6) (pooled sera)		231 ± 19	1.7	$P < 0.05$
Guinea pig	no. 1	47 ± 4	0.3	$P < 0.05$
	no. 2	37 ± 5	0.3	$P < 0.05$
	no. 3	7 ± 1	0.1	$P < 0.05$
Fetal calf (pooled sera)		129 ± 5	0.9	n.s.
Control culture*		137 ± 9	1.0	

* Control cultures contain 0.1 ml of phosphate buffered saline instead of serum in addition to 0.1 ml of L-cell conditioned medium.

Table 3. The effect of the addition of sera from patients with hematological disorders on the in vitro colony formation in the presence of L-cell conditioned medium.

Source of human serum	Number of colonies per plate	Relative enhancing activity	P value
Normal subjects			
no. 1	396 ± 28	3.8	$P < 0.05$
no. 2	338 ± 34	3.3	$P < 0.05$
no. 3	362 ± 18	3.5	$P < 0.05$
Aplastic anemia			
no. 1	493 ± 32	4.8	$P < 0.05$
no. 2	349 ± 16	3.4	$P < 0.05$
no. 3	358 ± 38	3.5	$P < 0.05$
Polycythemia vera			
no. 1	297 ± 22	2.9	$P < 0.05$
no. 2	356 ± 32	3.5	$P < 0.05$
no. 3	405 ± 28	3.9	$P < 0.05$
Chronic myelogenous leukemia			
no. 1	310 ± 18	3.0	$P < 0.05$
no. 2	506 ± 20	4.9	$P < 0.05$
no. 3	415 ± 38	4.0	$P < 0.05$
Acute myelogenous leukemia			
no. 1	410 ± 50	4.0	$P < 0.05$
no. 2	462 ± 35	4.5	$P < 0.05$
no. 3	296 ± 23	2.9	$P < 0.05$
Control culture*	103 ± 23	1.0	

* Control cultures contain 0.1 ml of phosphate buffered saline instead of serum in addition to 0.1 ml of L-cell conditioned medium.

Table 4. The effect of various pretreatment of normal human sera on the in vitro colony formation in the presence of L-cell conditioned medium.

Treatment of serum	Number of colonies per plate	Relative enhancing activity	P value
Dialysis			
Non-treated serum	158 ± 32	2.2	$P < 0.05$
Dialyzed serum	153 ± 25	2.1	$P < 0.05$
Redissolved precipitate	0	0	$P < 0.05$
Control culture*	73 ± 28	1.0	
Heating			
Non-treated serum	183 ± 9	3.3	$P < 0.05$
Heated serum			
No. 1 (50°C, 30 min.)	168 ± 16	3.0	$P < 0.05$
No. 2 (56°C, 30 min.)	173 ± 23	3.1	$P < 0.05$
No. 3 (60°C, 30 min.)	162 ± 6	2.9	$P < 0.05$
No. 4 (65°C, 30 min.)	18 ± 2	0.3	$P < 0.05$
Control culture*	56 ± 4	1.0	
Ether treatment			
Non-treated serum	235 ± 38	2.8	$P < 0.05$
Ether treated serum	88 ± 34	2.2	$P < 0.05$
Control culture*	85 ± 26	1.0	

* Control cultures contain 0.1 ml of phosphate buffered saline instead of serum in addition to 0.1 ml of L-cell conditioned medium.

Table 5. The effect of the addition of human albumin and Ig G globulin on the in vitro colony formation in the presence of L-cell conditioned medium.

Material	Number of colonies per plate	Relative enhancing activity
Human albumin		
12.5 mg/ml	71 ± 9	1.1
25.0 mg/ml	66 ± 14	1.0
50.0 mg/ml	69 ± 8	1.1
Human Ig G		
5.0 mg/ml	60 ± 10	1.0
10.0 mg/ml	56 ± 9	0.9
20.0 mg/ml	68 ± 18	1.1
Control culture*	63 ± 12	1.0

* Control cultures contain 0.1 ml of phosphate buffed saline instead of serum in addition to 0.1 ml of L-cell conditioned medium.

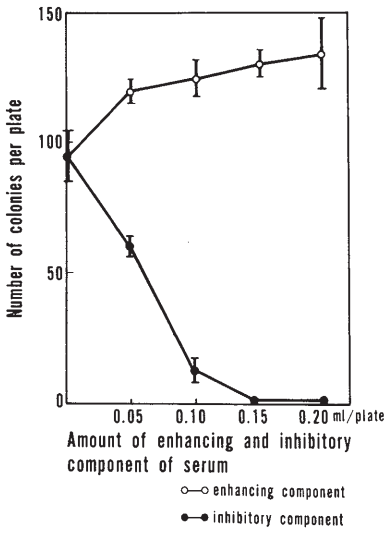

Fig. 3

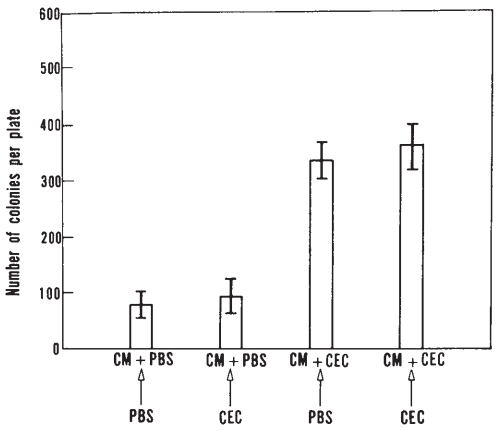

Fig. 4

Fig. 3. The effect of the serum colony enhancing and inhibitory component on the in vitro colony formation in the presence of L-cell conditioned medium.
The serum colony enhancing component is the concentrated material of collected fractions from no. 30 to 34 on Sephadex G-200 column chromatography.
The serum colony inhibitory component is that of collected fractions from no. 12 to 16.
Protein concentration of the serum colony enhancing component is 0.8 mg/ml and that of the serum inhibitory component is 1.8 mg/ml.

Fig. 4. The analysis of the interaction between the serum colony enhancing factor and CSF.
Bone marrow cells were preincubated with the serum colony enhancing component for 24 hours at 37°C. Thereafter, bone marrow cells were used for the assay of CFU-c. Letters in bottom row denote the materials within which bone marrow cells were preincubated, and those in top row denote the materials in underlayers of subsequent agar cultures.
CM: L-cell conditioned medium, PBS: phosphate buffered saline, CEC: colony enhancing component.

On the other hand, the higher molecular weight zone corresponding to Ig M showed the inhibitory effect as shown in Figure 2. Concentrated materials of collected fractions with the inhibitory activity showed their dose-response effect on the colony growth as illustrated in Figure 3.

Interaction between the serum colony enhancing factor and CSF

Figure 4 shows results of an experiment performed to analyse the interaction between the serum colony enhancing factor and CSF. The preincubation of marrow cells with the serum colony enhancing component for 24 hours did not enhance the colony growth in the L-cell conditioned medium containing agar cultures, and the coexistence of the active principle in human sera and CSF was required for the enhanced colony growth.

DISCUSSION

The present results demonstrated that human sera contained a factor (or factors) capable of enhancing the colony formation of mouse CFU-c stimulated by CSF. The sera from varieties of species except those from fetal calves and guinea pigs posses this enhancing activity on the colony growth of mouse marrow cells. The sera from patients with hematological disorders 'so far assayed' show the same degree of colony enhancing activities as those of normal human sera. This serum factor is non-dialysable, heat stable, non-extractable by ether and eluted in the lower molecular weight zone corresponding to albumin and Ig G on Sephadex G-200 column chromatography. It is thought to be most likely that this serum factor will be in the group of proteins having M.W. in the vicinity of 5 to 100,000. The cooperative action of the serum colony enhancing factor and CSF can be postulated as the possible mechanism of the enhanced colony growth by sera, although this mechanism is not yet revealed in detail.

Metcalf and his associates demonstrated the similar colony growth potentiating activity in mouse and human sera in the studies using mouse marrow cells as target cells.[6] They described the physico-chemical characteristics of the active factor in mouse sera was similar to those described in this report, although none was clarified as to the mechanism of the action.[6] Furusawa and his associates have shown that, in cultures using human marrow cells as target cells, the super-additive enhancing activity of human sera was in non-separated human marrow cells but not in non-adherent human marrow cells.[8] Thus, they postulated that the potentiating mechanism of human sera on the colony growth of human marrow cells may be due to the increased production of the endogenous CSF from adherent cells stimulated by sera added.[8] Francis and his associates described the same potentiating activity of human sera on the colony growth of human marrow cells which is dependent on the presence of bone marrow adherent cells.[9] However, this mechanism can not be applied to mouse marrow cell cultures employed in the present study, because no endogenous CSF production was demonstrated in this system. On the other hand, it has been shown that lymphocytes stimulated by various antigens can produce CSFs,[17] so the increased CSF production of mouse marrow lymphocytes stimulated by xenogeneic human sera can be another possible mechanism for the enhanced colony growth. However, syngeneic mouse sera could give rise to the enhanced colony growth, so this evidence has not been demonstrated in this system. The mechanism of the action of the colony enhancing factor in human sera dealt in this paper is different from those reported, therefore

the heterogeneity of the colony enhancing factor in human sera will be suggested.

In addition to sera, erythrocytes,[18] adenine compounds,[19] surface active agents[20] and lithium salts[21] have been reported to posses the potentiating effect on the in vitro colony growth. Erythrocytes were shown to stimualte the colony growth of CFU-c with different density profile from that induced by CSF alone.[22] It is postulated that surface active agents exert its enhancing activity through the increased responsiveness of CFU-c to CSF.[20] Lithium salts were considered to increase the systhesis of endogenous CSF in adherent marrow cells.[21]

Furthermore, present studies have shown that human sera contain a factor (or factors) capable of inhibitory the colony growth of mouse marrow cells which elutes in the higher molecular weight zone corresponding to Ig M on Sephadex G-200 column chromatography. Although some properties of this inhibitory factor in sera or urine have been reported by Chan,[23] Niskanen[24] and others,[25~28] the precise physico-chemical nature of this inhibitory factor and its interaction with the enhancing factor remain to be investigated in future.

As noted, the in vivo role of CSF as a humoral regulator on granulocyte and macrophage production has been investigated,[3~5] while the in vivo role of serum factors modifying CSF also will be the concern of this laboratory.

Furthermore, the results obtained tell us two practical points.[29] Firstly, these results suggest the development of a more sensitive assay method of CSF by adding an appropriate concentration of human sera in a culture system. Secondly, the caution should be paid in the interpretation of the value of CSF in sera measured by conventional assay systems, because it is necessary to make clear distinction between CSA and the serum enhancing effect.

SUMMARY

1. Sera from human, mice, rats and rabbits were shown to possess the enhancing activity of CFU-c colony growth from C_{57} BL/6 mouse marrow cells in the presence of L-cell conditioned medium. This enhanced colony growth by human sera was also shown in the presence of conditioned medium prepared from human peripheral blood leukocytes, C_{57} BL/6 mouse whole embryo and sera from E. coli endotoxin-injected C_{57} BL/6 mice.

2. The active principle in human sera was heat-stable, non-dialysable, not extractable by ether and separated in the lower molecular weight zone corresponding to albumin and Ig G by gel filtration of Sephadex G-200. Neither pure human albumin nor Ig G showed any enhancing effect on the colony formation.

3. The inhibitory effect of the colony growth was demonstrated in the higher molecular weight zone corresponding to Ig M of human sera on Sephadex G-200 gel filtration.

4. The cooperative action of the active principle in human sera and CSF is thought to be required for the enhancement of the colony growth, because the pretreatment of marrow cells with the active principle in human sera did not show any effect on the colony growth, and the coexistence of the active principle in human sera and CSF was required for the enhanced colony growth.

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