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COLONY-STIMULATING ACTIVITY IN CULTURES OF HUMAN SPLEEN AND BONE MARROW CELLS

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ABSTRACT

We found that human bone marrow cells formed mononuclear phagocyte clusters and colonies in liquid culture with or without conditioned medium. In this study, we prepared media from human spleen or bone marrow cells in several different conditions and assessed their colony-stimulating activities as well as the sensitivities of human spleen or bone marrow colony-forming cells to these conditioned media. Conditioned media from human spleen cell cultures showed distinct cluster-stimulating activity to human bone marrow cells but those from human bone marrow cell cultures did not. On the other hand, conditioned media from human spleen cell cultures showed distinct colony-inhibiting rather than -stimulating activity to human spleen cells with high plating efficiency ($11-350/4 \times 10^6$) compared with those from human bone marrow cell cultures. To human spleen cells with low plating efficiency ($1-10/4 \times 10^6$), conditioned media from human bone marrow cell cultures showed distinct cluster-stimulating activity compared with those from human spleen cell cultures.

Running Head: Colony-stimulating activity of human spleen and bone marrow cells

INTRODUCTION

Many studies on granulocyte-monocyte (GM) colony formation of murine and human bone marrow cells in soft agar have been conducted since the reports of Bradely and Metcalf (2) and Pluznik and Sachs (3) in 1966. Stewart and his colleagues reported macrophage colony formation of murine peritoneal exudate cells in liquid culture in 1975 (4). Yokochi and his colleagues reported macrophage colony formation of murine bone marrow and spleen cells in liquid culture in 1980 (5). In these reports, GM or macrophage colony formation required a feeder layer or conditioned medium. On the other hand, Moore and Williams reported hemopoietic colony formation in soft agar occurring spontaneously in mass cultures of bone marrow cells obtained from a number of species (6). We also found that human spleen and bone marrow cells formed mononuclear phagocyte colonies without a feeder layer or conditioned medium (1). Furthermore, we observed that spontaneous colony formation by human spleen and bone marrow cells was influenced by culture media of human spleen or bone marrow cells. In this study, we examined the specificity of conditioned media derived from both spleen and bone marrow cells to cluster- or colony-forming cells of mononuclear phagocytes in hemopoietic organ and its candidate, namely, bone marrow and spleen in humans.

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MATERIALS AND METHODS

Cell preparation and culture

Human spleen cells were obtained from patients with portal hypertension, gastric cancer, and other diseases when splenectomy was required for therapy. Single cell suspension was prepared as described previously (1). Human bone marrow cells were obtained from patients with lung cancer or other diseases when removal of rib was required for thoracotomy. The rib was crushed and flushed with sterile medium. The medium with bone marrow was vigorously pipetted and filtrated through stainless steel mesh. Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (GIBCO Laboratories, N.Y., USA), 2mM L-glutamine, and 5×10^{-5}M 2-mercaptoethanol was used as the culture medium.

Cluster-colony formation in liquid culture

Four million spleen cells or 2×10^6 bone marrow cells were seeded in culture dishes (Falcon 35 mm) containing 2 ml culture medium with or without 0.5 ml conditioned medium and were incubated in triplicate for 7 days at 37°C in 5% CO_2-humidified atmosphere. Clusters and colonies formed on the bottom of the culture dish were fixed with 99% ethanol and stained with Giemsa's solution. Aggregations composed of 10–30 cells were counted as clusters and those composed of more than 30 cells, as colonies (1).

Preparation of conditioned medium

Spleen or bone marrow cells were cultured at various concentrations of nucleated cells in Falcon 3042 culture flasks containing 50 ml culture medium for 5 days at 37°C in 5% CO_2-humidified atmosphere. The cultured cell suspension was centrifugated at 2000 rpm for 10 min and the supernatant was stored at –20°C until use. Five-day culture of the cells decreased their viability from 90–80% at the start of culture to 70–60%.

Statistics

Data were evaluated by paired t-test.

RESULTS

Mononuclear phagocyte cluster and colony formation by human bone marrow cells in liquid culture

Most tested bone marrow cells ($1–4 \times 10^6$/dish) formed cell aggregations regardless of the presence of conditioned medium in liquid culture after incubation for 7 days at 37°C in 5% CO_2-humidified atmosphere. As shown in Fig. 1, the aggregations consisted of both non-phagocytic round cells and elongated cells phagocytizing formalin-fixed sheep red blood cells, which is similar to the mononuclear phagocyte clusters or colonies of human spleen cells formed in liquid culture described previously (1). These aggregations increased depending on the number of seeded bone marrow cells. The relationship between spontaneous cluster or colony formation and the number of seeded cells was sigmoid (Fig. 2-a) but that between conditioned-medium-induced cluster formation and the number of seeded cells was linear within a range of the number of seeded cells (Fig. 2-b). These aggregations did not appear at 4 days but at 7 days after the start of incubation. More than 10 days of incubation decreased the numbers (Fig. 3).

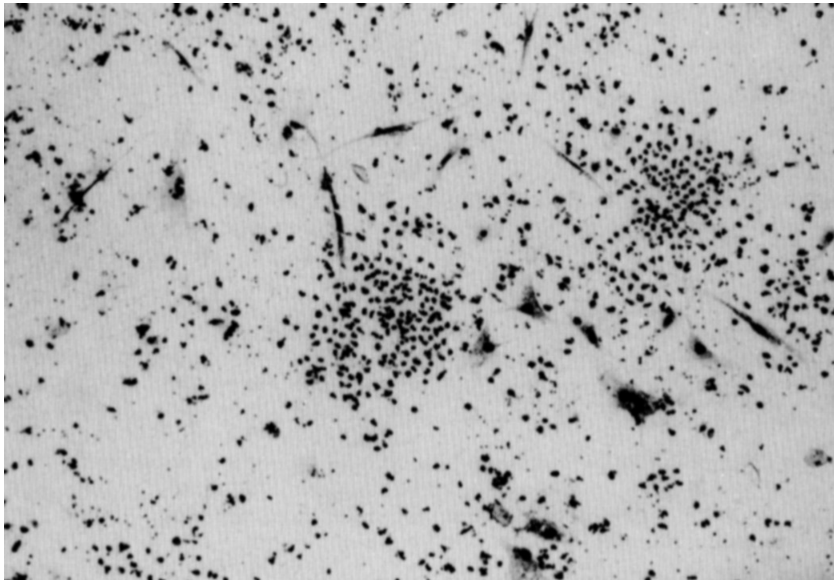

Fig. 1-a

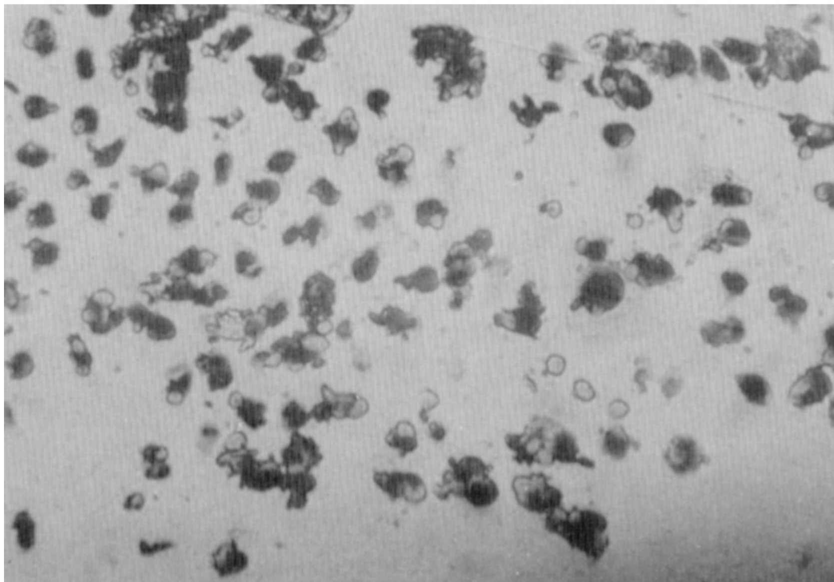

Fig. 1-b

Fig. 1 Morphology of mononuclear phagocyte colony of human bone marrow cells.
a: × 100, b: × 400

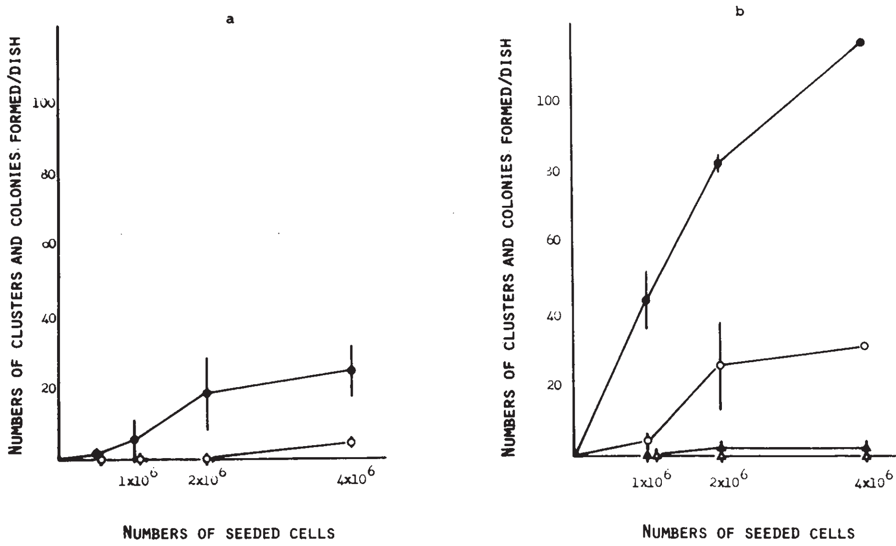

Fig. 2 Dose-dependent cluster-colony formation by human bone marrow cells.
a: spontaneous cluster (●) and colony (○) formation.
b: cluster (● ▲) and colony (○ △) formation with (● ○) or without (▲ △) conditioned medium.

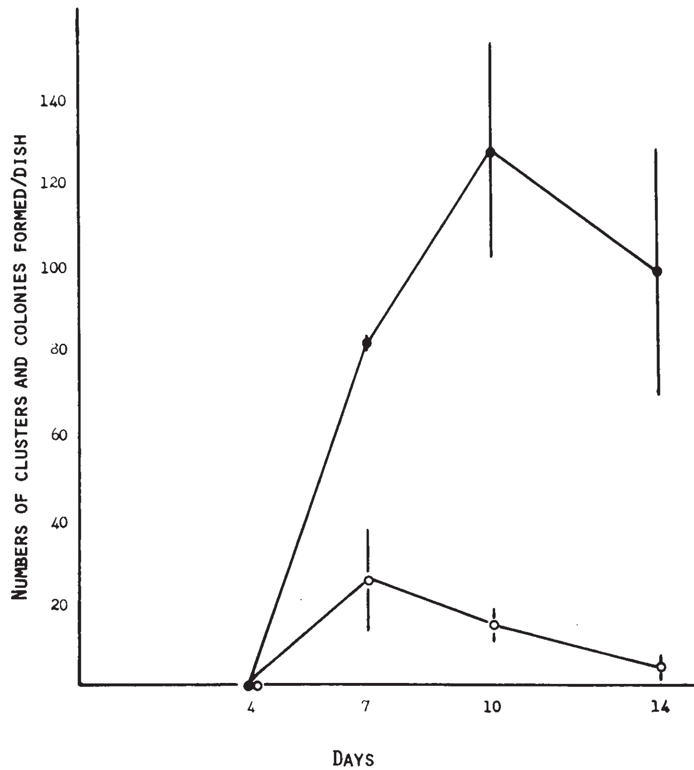

Fig. 3 Kinetics of cluster and colony formation.
Human bone marrow cells cultured with human-spleen-conditioned medium formed clusters (●) and colonies (○).

Effect of numbers of cultured human spleen- or bone-marrow-conditioning cells on cluster- or colony-stimulating activity of the conditioned medium

Effect of numbers of cultured spleen- or bone marrow-conditioning cells on cluster- or colony-stimulating activity of the conditioned medium was studied (Table 1). The cluster- or colony-stimulating activity of conditioned medium depended on the numbers of spleen- or bone-marrow-conditioning cells cultured. However, the number (1×10^7/ml) of conditioning cells cultured was too large to show cluster- or colony-stimulating activity fully. Therefore, we used medium conditioned with 5×10^6/ml spleen or bone marrow cells, unless otherwise specified.

Difference between human spleen- and bone-marrow-conditioned media on their cluster- or colony-stimulating activities to human spleen and bone marrow cluster or colony formation

As shown in Table 2, spleen-conditioned media showed cluster- and colony-stimulating activities in 42% (in cluster) and 25% (in colony) out of 24 pairs consisting of 7 lots of bone-marrow-cluster- and colony-forming cells and 11 lots of spleen-conditioned medium, while bone-marrow-conditioned media showed only cluster-stimulating activities in 40% out of 5 pairs consisting of 3 lots of bone-marrow-cluster- and colony-forming cells and 3 lots of bone-marrow-conditioned medium. In this experiment, the bone-marrow-cluster- and colony-forming cells used had low plating efficiencies ($1-10/2 \times 10^6$). Moreover, although numbers of tested pairs were small, bone-marrow-conditioned media showed cluster- and colony-inhibiting activities in 50% (in cluster) and 50% (in colony) out of 4 pairs consisting of 2 lots of bone-marrow-cluster- and colony-forming cells and 2 lots of bone-marrow-conditioned medium while spleen-conditioned media showed lower frequency of detected cluster- and colony-inhibiting activities in 29% (in cluster) and 18% (in colony) out of 17 pairs consisting of 5 lots of bone-marrow-cluster- and colony-forming cells and 9 lots of spleen-conditioned medium. In this experiment, bone-marrow-cluster- and colony-forming cells used had high plating efficiencies ($11-50/2 \times 10^6$). Alternatively spleen-conditioned media showed significant cluster-stimulating activities to bone marrow cells compared with bone-marrow-conditioned media (Table 6).

To spleen cells with low plating efficiencies ($1-10/4 \times 10^6$), bone-marrow-conditioned media showed cluster- and colony-stimulating activities in 30% (in cluster) and 10% (in colony) out of

Table 1. Effect of numbers of human spleen- or bone-marrow-conditioning cells cultured on cluster- or colony-stimulating activity of the conditoned medium.

Exp.	Origin of cluster- and colony-forming cells	Origin of conditioning cells	Numbers of conditioning cells/ml	Numbers of clusters formed/dish	Numbers of colonies formed/dish
1	Spleen	Spleen	0	188(37)	23(2)
			2×10^6	229(34)	33(16)
			5×10^6	214(64)	54(7)
			1×10^7	158(10)	113(11)
2	Spleen	Bone marrow	0	4(2)	2(2)
			5×10^6	45(7)	4(2)
			1×10^7	12(6)	3(2)
3	Bone marrow	Spleen	0	0(0)	0(0)
			2×10^6	40(7)	24(2)
			5×10^6	62(15)	48(21)
			1×10^7	77(16)	59(10)

Table 2. Effect of human spleen- or bone-marrow-conditioned medium (C.M.) on spontaneous cluster or colony formation of human bone marrow cells.

Plating efficiency of spontaneous cluster and colony formation	Origin of C.M.	Percentage of pairs showing cluster or colony stimulation[4)]		Percentage of pairs showing cluster or colony inhibition[4)]		
		Cluster	Colony	Cluster	Colony	
Low (1–10/2 x 10^6)	Bone marrow	40	0	0	0	1) 5 2) 3 3) 3
	Spleen	42	25	38	0	1) 24 2) 7 3) 11
High (11–50/2 x 10^6)	Bone marrow	50	50	50	50	1) 4 2) 2 3) 2
	Spleen	59	53	29	18	1) 17 2) 5 3) 9

1) Numbers of tested pairs consisting of both cluster- and colony-forming cells and C.M.
2) Numbers of tested lots of cluster- and colony-forming cells.
3) Numbers of tested lots of C.M.
4) The stimulation and inhibition were judged to be positive when the difference from the mean number of clusters and colonies formed spontaneously was statistically significant.

Table 3. Effect of human spleen- or bone-marrow-conditioned medium (C.M.) on spontaneous cluster or colony formation of human spleen cells.

Plating efficiency of spontaneous cluster and colony formation	Origin of C.M.	Percentage of pairs showing cluster or colony stimulation[4)]		Percentage of pairs showing cluster or colony inhibition[4)]		
		Cluster	Colony	Cluster	Colony	
Low (1–10/4 x 10^6)	Bone marrow	30	10	0	0	1) 10 2) 4 3) 5
	Spleen	21	0	0	0	1) 29 2) 9 3) 12
High (11–350/4 x 10^6)	Bone marrow	0	14	38	7	1) 14 2) 4 3) 4
	Spleen	0	0	50	75	1) 8 2) 4 3) 2

1) Numbers of tested pairs consisting of both cluster- and colony-forming cells and C.M.
2) Numbers of tested lots of cluster- and colony-forming cells.
3) Numbers of tested lots of C.M.
4) The stimulation and inhibition were judged to be positive when the difference from the mean number of clusters and colonies formed spontaneously was statistically significant.

10 pairs consisting of 4 lots of spleen-cluster- and colony-forming cells and 5 lots of bone-marrow-conditioned medium while spleen-conditioned media showed only cluster-stimulating activities in 21% out of 29 pairs consisting of 9 lots of spleen-cluster- and colony-forming cells and 12 lots of spleen-conditioned medium (Table 3). Moreover, spleen-conditioned media showed higher cluster- and colony-inhibiting activities to spleen cells with high plating efficiencies ($11-350/4 \times 10^6$) in 50% (in cluster) and 75% (in colony) out of 8 pairs consisting of 4 lots of spleen-cluster- and colony-forming cells and 2 lots of spleen-conditioned medium compared with bone-marrow-conditioned media. Alternatively, bone-marrow-conditioned media showed significant cluster-stimulating activities to spleen cells with low plating efficiencies compared with spleen-conditioned media (Table 4). To spleen cells with high plating efficiencies, spleen-conditioned media showed significant colony-inhibiting activities compared with bone-marrow-conditioned media (Table 5).

These results suggested that cluster- or colony-forming cells of mononuclear phagocytes in both human spleen and bone marrow had different susceptibilities to media conditioned by component cells of each organ.

DISCUSSION

We found that aggregations were formed by human bone marrow cells in liquid culture after incubation for 7 days at 37°C in 5% CO_2-humidified atmosphere regardless of the presence of conditioned medium. The morphology, dependency on numbers of seeded cells, and kinetics of appearance of the aggregations were similar to those of human spleen mononuclear phagocyte clusters and colonies formed in liquid culture described previously (1); i.e., aggregations hardly appeared by 4 days of the culture, their numbers increased proportionately to those of seeded cells in the presence of conditioned medium, and they were composed of both non-phagocytic round cells and phagocytic elongated cells. Moreover, mononuclear phagocyte cluster and colony formation by human spleen cells was blocked by low doses of mitomycin-C which is known to inhibit DNA synthesis selectively (1). Therefore, we considered the aggregations formed by human bone marrow cells in this study to be clusters or colonies.

The role of colony-stimulating factor (CSF) still remains controversial in vivo and in long-term culture of bone marrow (9). On the other hand, several kinds of inhibitors to hemopoietic stem cell proliferation were reported (10–14). Murine bone marrow cells were used in general for determining colony-stimulating activity (CSA) in human serum and urine (7). However, another author suggested that a delicate difference existed in the interspecies compared with the intraspecies CSA (6, 8). Therefore, it is better to use human hemopoietic cells for determining human CSA.

Human-spleen-conditioned medium was used for formation of granulocyte colonies by normal human bone marrow cells in soft agar culture by Paran and his colleagues in 1970 (15). Their human-spleen-conditioned media induced colonies of macrophages or granulocytes from murine bone marrow cells as well as colonies of granulocytes from human bone marrow cells. Human-spleen-conditioned media prepared in this study induced clusters and colonies from murine (data not shown) as well as human bone marrow cells. However, they were not granulocyte colonies but mononuclear phagocyte clusters and colonies. This difference from the results reported by Paran and his colleagues may be attributed to the difference of actual CSA in both conditioned media or to the difference of both culture systems.

We found that colony-inhibiting activity to human spleen cells as well as cluster-stimulating activity to human bone marrow cells existed in human-spleen-conditioned medium (Table 2, 3, 5 and 6). It is interesting for human-spleen-conditioned medium to inhibit human spleen cell

Table 4. Comparison between human spleen- and bone-marrow-conditioned medium (C.M.) of their effect on spontaneous cluster or colony formation of human spleen cells with low plating efficiency.

Donor of cluster- and colony-forming cells	Pairs of both spleen- and bone-marrow-C.M.[1]					
	Spleen-C.M.			Bone-marrow-C.M.		
	Donor of conditioning cells	Difference from spontaneous cluster or colony formation[2]		Donor of conditioning cells	Difference from spontaneous cluster or colony formation[2]	
		Cluster	Colony		Cluster	Colony
T.Y.	K.M.	+11	−1	K.O.	+93	+8
	H.Y.	+4	−2	S.U.	+81	+4
	T.F.	−2	−1	X.T.	+41	+2

1) Difference between spleen- and bone-marrow-C.M. was significant in cluster formation ($t=5.465$, $0.02<p<0.05$) but not in colony formation ($t=2.450$, $0.1<p<0.5$).
2) The mean number of spontaneous clusters or colonies was subtracted from that of clusters or colonies in cultures with indicated C.M., respectively.

Table 5. Comparison between human spleen- and bone-marrow-conditioned medium (C.M.) of their effect on spontaneous cluster or colony formation of human spleen cells with high plating efficiency.

Donor of cluster- and colony-forming cells	Pairs of both spleen- and bone-marrow-C.M.[1]					
	Spleen-C.M.			Bone-marrow-C.M.		
	Donor of conditioning cells	Difference from spontaneous cluster or colony formation[2]		Donor of conditioning cells	Difference from spontaneous cluster or colony formation[2]	
		Cluster	Colony		Cluster	Colony
C.S.	T.K.	−98	−72	K.M.	−92	+107
	T.F.	+26	−53	S.U.	−76	+108
H.K.	T.K.	−52	0	M.I.	+3	0
	T.F.	−13	0	K.O.	+10	0
K.K.	T.K.	−47	−117	K.M.	−8	+68
	T.F.	+52	−107	S.U.	+31	+3
T.O.	T.K.	−63	−54	M.I.	−58	−47
	T.F.	−55	−54	S.U.	+8	−11

1) Difference between spleen- and bone-marrow-C.M. was significant in colony formation ($t = -2.952$, $0.02<p<0.05$) but not in cluster formation ($t = 0.574$, $0.5<p$).
2) The mean number of spontaneous clusters or colonies was subtracted from that of clusters or colonies in cultures with indicated C.M., respectively.

colony formation (Table 3, 5) and to stimulate preferentially human bone marrow cell cluster formation (Table 2, 6). Further, it is interesting for human-bone-marrow-conditioned medium to stimulate preferentially human spleen cell cluster formation (Table 3, 4) but not human bone marrow cell cluster and colony formation. We should note whether the counterpart of paired sample for statistical analysis represents each statistical population consisting of either normal-spleen-or bone-marrow-derived conditioned media. However, the activity itself of conditioned medium may be of no importance but the specificity of the activity to cluster- or colony-forming cells may be of importance in this study. Conditioned media derived from bone marrow cells

Table 6. Comparison between human spleen- and bone-marrow-conditioned medium (C.M.) of their effect on spontaneous cluster or colony formation of human bone marrow cells.

Donor of cluster- and colony-forming cells	Pairs of both spleen- and bone-marrow-C.M.[1]					
	Spleen-C.M.			Bone-marrow-C.M.		
	Donor of conditioning cells	Difference from spontaneous cluster or colony formation[2]		Donor of conditioning cells	Difference from spontaneous cluster or colony formation[2]	
		Cluster	Colony		Cluster	Colony
K.M.	H.Y.	+138	+29	X.T.	+7	0
	K.M.	+67	+28	S.U.	+5	0
X.T.	T.F.	0	0	S.U.	0	0
X.F.	T.K.	+62	+48	S.U.	+2	0
	T.F.	+20	+1	K.M.	0	0
T.A.	H.Y.	+154	+34	S.U.	+133	+64
	T.F.	+103	+5	X.T.	+36	+17

1) Difference between spleen- and bone-marrow-C.M. was significant in cluster formation ($t=3.168$, $0.01<p<0.02$) but not in colony formation ($t=0.880$, $0.5<p$).
2) The mean number of spontaneous clusters or colonies was subtracted from that of clusters or colonies in cultures with indicated C.M., respectively.

of two patients (S.U. and X.T.) showed distinct cluster-stimulating activity to spleen cells but not to bone marrow cells. In contrast, conditioned media derived from spleen cells of two patients (T.F. and H.Y.) showed cluster-stimulating activity to bone marrow cells but not to spleen cells.

From the above findings, it was suggested that both conditioned media from spleen and bone marrow possessed cross-specificity to each cluster- or colony-forming cells, although the possibility that the effect was attributed not to normal but to spleen and bone marrow in a pathological state could not be ruled out. As far as we are aware, such reports of human spleen and bone marrow interacting with each other by cross-specificity of each CSA to each cluster- or colony-forming cells are few. We remember that Hasewaga and his colleagues suggested the dynamic interaction between spleen and bone marrow by analysis of the kinetics of macrophage colony-forming cells after X-irradiation or administration of cyclophosphamide in murine system (16). It is conceivable that both human spleen and bone marrow cluster- or colony-forming cells are heterogeneous to each other and that they have different responsiveness to both human spleen and bone marrow CSA which may also be heterogeneous to each other since heterogeneity of human bone marrow colony-forming cells (17) as well as human CSA (8, 17) has been reported. Further investigation is required on the heterogeneity of cluster- or colony-forming cells and CSA, of both human spleen and bone marrow.

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