Tissue Culture of Calf Bone Marrow. Requirements of Serum and Conditioned Media (36803)

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The agar culture method for growing colonies of granulocytes and macrophages from bone marrow cells has provided a technique for assaying a humoral factor, the colony-stimulating factor (CSF), which is found in plasma (1) and urine (2). This factor appears to be similar to CSF produced by feeder layers of bone marrow (3), peripheral leukocytes (4) or kidney cells (5). In general, colonies of bone marrow do not form unless this stimulant is continually present. Shadduck and Nunna (1) found elevation of CSF in serum only during experimentally induced neutropenia, favoring the view that CSF is a humoral regulator of myelopoiesis.

Although serum does not usually stimulate bone marrow colonies, Bradley, Metcalf and Robinson (6) found CSF in serum of AKR mice when leukemia developed. Bradley and Siemienowicz (7) also reported stimulation of rat bone marrow by normal rat serum. In both cases, however, fetal bovine serum was used in the culture. The question of whether normal serum can stimulate bone marrow without interaction with some other factor has not been resolved.

We have developed a liquid tissue culture system for the study of bone marrow stimulants. In this system proliferation begins by the second day and reaches a peak on the third or fourth day (unpublished data) which is equal to or greater than that of the original specimen. Macrophage transformation is prominent by day two and is complete by day seven. We have found that conditioned media or heterologous sera are not required for marrow growth. However, conditioned medium can be shown to have a synergistic effect with serum when serum is limiting.

Materials and Methods. One milliliter of bone marrow aspirate from an unanesthetized calf 1–8 wk old was dispersed in 200 ml of cold Eagle's minimal essential medium (MEM) without anticoagulant and collected by centrifugation. The marrow pellet was broken up in a small amount of supernatant and dispersed by passage through a 22 gauge and then a 25 gauge needle. Cultures (5 ml) were prepared containing 0–80% serum (v/v), 20–100% MEM, 2.5×10^6 marrow cells and plated on 60 mm petri dishes. Cultures were incubated at 37° in 5% CO_2, 95% air saturated with water vapor. Tritiated thymidine (1.9 Ci/mmole) 1 μCi/ml was added after 3 days' incubation. Two hours later the culture was transferred to a centrifuge tube with the aid of a rubber policeman, washed once with acetic methanol 1:3, precipitated 1 hr with cold 10% trichloroacetic acid (TCA), washed three times with ice-cold 5% TCA, dissolved in Soluene and counted in a liquid scintillation counter using Bray's mixture; cpm were converted to dpm by external standardization. Pairs of cultures were averaged for each data point. Data was excluded from analysis if either culture result was >15% from the average of the pair.

Conditioned medium was prepared from the peripheral blood leukocytes of patients with polycythemia, from normal calf peripheral blood leukocytes, or from calf bone marrow by the method of Chervenick and Boggs (8).

Dose–response curves of fetal, newborn calf (2–8 wk old) and adult bovine serum were obtained by varying the serum concentration from 0 to 80% (v/v) in MEM. Two to three different sera were studied in each age group. Inhibition at high and low concentrations

TABLE I. Dose–Response Plot of Serum of Cows of Varying Age on the Incorporation of Tritiated Thymidine into Calf Bone Marrow Cells *in Vitro*.

		% Serum concn (v/v)						
		0	10	20	30	40	60	80
Fetal	$\overline{X}$	1.3[a]	44.7	55.8	64.2	63.8	22.4	2.2[b]
	SEM	1.1	13.6	16.6	22.6	18.9	9.5	0.9
	N	5	3	4	4	5	4	4
Calf	$\overline{X}$	0.2	20.1	36.9	54.6	59.7	51.4	49.4
	SEM	0.07	4.9	10.4	12.0	16.5	29.6	25.7
	N	5	3	3	5	5	3	4
Adult	$\overline{X}$	0.2	13.8	34.7	70.6	86.6	76.5	60.4[b]
	SEM	0.08	4.4	7.9	—	8.4	12.3	14.3
	N	5	3	3	2	4	3	3

[a] dpm $\times$ 10^{-3}/culture.
[b] $p = < .01$.

was exploited in further experiments.

The recovery of marrow from serum inhibition was studied by incubating cells in 80% fetal bovine serum for 3, 5, or 6 days, then replacing with 40% serum. The cultures were decanted, the loose cells washed with 40% serum in MEM and replaced in the original petri dishes. Pairs of cultures were removed daily following 2 hr incubation with tritiated thymidine to determine the rate of recovery of DNA synthesis.

A serum-deprived system was used to test the effect of conditioned medium. Serum (7%) in MEM was mixed with concentrations of conditioned medium from 0 to 60% (v/v). Conditioned medium was also serially diluted with MEM alone to determine its effect in the absence of added serum. Then the effect of 7% serum mixed with optimum concentrations of conditioned medium was compared with the effect of 7% serum or conditioned medium in separate cultures. Finally, serum was serially added to optimum concentrations of conditioned medium and compared with optimum serum concentration alone.

Results. Table I shows that the addition of serum to MEM resulted in peak thymidine incorporation at 30–40% serum (v/v) regardless of the age of animal donating serum. Fetal bovine serum, however, differed from the others by showing marked inhibition of incorporation at high concentrations. The difference between fetal calf serum and cow serum at 80% concentration was significantly different ($p < .01$). The reversibility of the inhibition by 80% fetal calf serum was readily demonstrated by replacing the inhibitory 80% concentration with 40% serum. Figure 1 shows that DNA synthesis was markedly slowed by the third day in the presence of 80% fetal bovine serum. Restoration of growth occurred promptly when the medium was changed to 40% serum, but was less successful when cultures were incubated in 80%

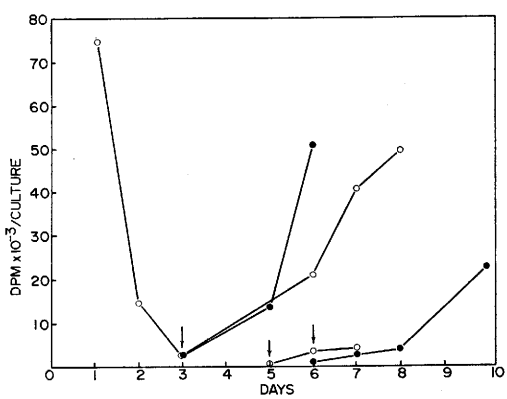

FIG. 1. The effect of 80% fetal bovine serum on subsequent growth of calf bone marrow cells. Cells were grown in 80% fetal bovine serum, 20% MEM for periods up to 6 days, then replaced (arrow) by 40% calf serum, 60% MEM. The decline in DPM to less than 10% of initial values was reversed. (●) Expt I; (○) Expt. II.

TABLE II. Effect of Serum With and Without Conditioned Medium on the Incorporation of Tritiated Thymidine in 3-Day-Old Calf Bone Marrow Cultures.[a]

	% Conditioned media (v/v)							
	0	5	10	15	20	40	60	80
With 7% serum								
$\bar{X}$	13,013[b]	36,699	39,915	32,888	19,986	3023	719	57
SEM	1208	7125	7434	7028	4144	1464	529	30
N	6	6	6	6	6	6	6	6
Without 7% serum								
$\bar{X}$	50[b]	4366	4388	3614	1980	594	497	40
SEM	3.6	1075	1312	1563	1388	516	304	12
N	6	6	6	6	6	6	6	6
p	$<.001$	$<.01$	$<.01$	$<.025$	$<.025$	$>.05$	$>.05$	$>.05$

[a] Dose–response plot using two sera and four conditioned media.
[b] dpm/culture.

serum for more than 3 days.

At the lower end of the serum dose–response curve, augmentation of DNA synthesis was demonstrated by the addition of conditioned media. Table II shows the dose–response plot of marrow cells cultured with concentrations of conditioned medium from 0 to 80% with or without 7% serum. This demonstrates that the presence of conditioned medium significantly increased the thymidine incorporation by 7% serum. The peak of activity for conditioned medium with or without added serum was at 10%.

TABLE III. Effect of Conditioned Media in Combination with Suboptimal Serum Concentrations.[a]

1 7% Serum	2 5–15% Cond. media	1 + 2	3 7% Serum + cond. media
15,458[b]	2974	18,432	45,339
15,553	2645	18,198	44,138
15,553	1998	17,551	40,439
12,525	9001	21,526	58,717
9497	10,964	20,461	26,757
9497	4344	13,841	17,527
		$p < .025$	

[a] Demonstration of synergistic effect. Pairs of calf marrow cultures were incubated with 7% calf serum (media 1), conditioned media 5–15% (media 2) or the combination for 3 days (media 3). Thymidine incorporation into DNA was compared for the sum of serum and conditioned medium separately (media 1 + 2) and combined (media 3). The difference was significant $p < .025$. Three conditioned media, and two sera obtained from the same calf were used.
[b] dpm/culture.

Table III demonstrates that cultures containing both 7% serum and 5–15% conditioned medium incorporated significantly more thymidine than was predicted from the sum of dpm from cultures containing either 7% serum or 5–15% conditioned medium ($p < .025$). This suggested a synergistic effect between conditioned medium and serum.

Using 10% conditioned medium as optimal, we then varied the concentration of calf serum and compared the result with 40% calf serum alone. Table IV shows that there was no significant difference between the highest dpm obtained with 10% conditioned medium and concentrations of calf serum up to 60%, compared with the counts in the same culture obtained with 40% calf serum alone.

Discussion. Calf bone marrow tissue culture appears to be a simpler system than culture systems previously described, requiring only homologous or autologous serum and chemically defined nutrient medium. Thirty to 40% serum was optimal and at the optimum there was no significant difference between fetal, calf, and adult cow serum. Metcalf, Bradley, and Robinson (5) found smaller colonies when bovine serum was sub-

TABLE IV. Dose–Response Plot of 10% Conditioned Medium and Varying Amounts of Calf Serum Compared with 40% Serum Alone.[a]

% Serum:	0	10	20	40	60	80	No C.M. 40%
	136[b]	2577	3516	3703	*4726*		*8987*
	136	884	*3133*	2749	1379		*2098*
	126	1071	*3245*	3282	2505		*4210*
	1184	37,130	*89,718*	51,925	59,621	32,685	*65,723*
	7343	70,222	*71,862*	46,322	41,018	27,664	*46,079*
	—	40,113	55,074	66,857	*70,592*	65,834	*72,495*
	208	2590	3208	6053	*10,548*		*8987*
	208	2278	4067	*4675*	2622		*2098*
	208	221	3777	4265	*4407*		*4210*

[a] Thymidine incorporation in 3-day-old calf bone marrow cultures. Italicized values are highest dpm of serum + conditioned medium, compared with 40% serum alone. The difference between the italicized values was not significant, $p > .05$. Two conditioned media and four sera were used in these experiments.

[b] dpm/culture.

stituted for fetal calf serum but the differences were probably not significant.

Fetal bovine serum inhibited thymidine incorporation at both high and low concentrations. We demonstrated reversibility of inhibition at 80% serum by replacement with 40% serum. Inhibition by 7% serum was used to study the effect of conditioned media.

At low concentrations of serum, conditioned medium was observed to have a significant effect: dpm of cultures containing both 7% serum and 10% conditioned medium were significantly greater than the sum of dpm from cultures containing either 7% serum or 10% conditioned medium. This suggested an interaction between serum and conditioned medium which could only be seen when serum was limiting. In their studies of rat marrow grown with rat serum and fetal bovine serum, Bradley and Siemienowicz (7) also suggested that two components were involved in the stimulation of marrow growth.

At optimal serum concentrations, conditioned medium did not show a stimulatory effect. The fact that 40% serum obscured the effect of added conditioned medium may be explained either by the presence of the conditioned media factor in normal calf serum or its generation by the cultured marrow cells in the assay system. The solution awaits further study.

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