Recovery of Leukopoietic Activity from Organ Perfusion Medium (34230)

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A recent method has been developed for the colony growth of bone marrow cells *in vitro* (1). In this system, with an appropriate stimulus, single cell suspensions of bone marrow cells in agar-gel medium give rise to granulocytic and mononuclear cell colonies (2). A variety of sources have been used as stimulants for colony growth. Among these are kidney and whole mouse embryo cell feeder layers (1, 3), "conditioned medium" from these cell sources, (4, 5) and animal and human serum and urine (6–8). There would appear to be a common or similar factor in all of these sources which is the major stimulatory material necessary for colony growth. In serum and urine this factor has been identified as a protein or glycoprotein with a molecular weight of approximately 60,000 which migrates electrophoretically as an alpha-globulin (9, 10). It has been suggested that this factor may be a true leukopoietic material stimulating maturation and production of granulocytes in the bone marrow *in vivo* (9).

The finding of this material in serum and urine has prompted a search for tissue sources. The present studies demonstrate that a factor similar to that previously described in body fluids and conditioned medium and capable of stimulating granulocyte colony growth *in vitro*, can be detected in tissue culture medium used to perfuse isolated whole porcine spleens and livers.

Methods and Materials. Spleens and livers were removed aseptically from heparinized 12–18 kg Hampshire pigs. Blood was washed from the organs with two liters of 0.9% sodium chloride containing 10,000 units of heparin, 60 mg of procaine hydrochloride, and 7 meq of sodium bicarbonate/liter. Following washout the organs were perfused separately in a sterile closed perfusion apparatus previously described (11).

Perfusing medium was McCoy's 5A with 10% pooled pig serum (Colorado Serum Laboratories). Oxygenation of the perfusate was achieved by the use of a silastic membrane oxygenator keeping the arterial pO_2 at approximately 250 mm Hg (12). Using this system, pig spleens and livers have been successfully perfused for up to 1 week (11).

The perfusing medium was not changed. After varying durations, aliquots of the medium were collected through a 3-way stopcock and tested against samples of the original uncirculated medium to stimulate and sustain bone marrow colony growth *in vitro*.

The system used for colony growth of bone marrow cells is a modification of that previously described (2). Bone marrow from the femurs of 2–3-month-old C57/Bl mice was prepared as a single cell suspension in McCoy's 5A medium. Perfusate medium to be tested was mixed in a 9:1 concentration with boiled 3% agar (Bacto-agar, Difco), giving a final agar concentration of 0.3%. To this mixture bone marrow cells were added to a concentration of 75,000 nucleated cells/ml. One-ml aliquots of ths mixture were placed in 35-mm Falcon plastic petri dishes. Unlike previous studies no stimulatory material, *i.e.*, serum, urine, or conditioned medium was added to the plates. The medium was allowed to gel at room temperature, and the plates were incubated at 37° under humidified conditions with a constant flow of 5% CO_2 in air. Colony counts were done after 7-days incubation with a dissecting microscope at 10× magnification. Previous studies

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have shown a linear relationship between the number of colonies detected and the amount of active material present (2). Thus by counting the number of colonies, a quantitative estimate was obtained of the colony-stimulating activity in test samples. Each experiment served as its own control since individual batches of porcine serum used in culture medium varied as to their ability to stimulate and sustain colony growth.

For microscopic study colonies were removed from the agar medium with a finely drawn Pasteur pipette and prepared as aceto-orcein stained wet mounts as previously described (2).

In dialysis studies 50 ml of medium was dialyzed in Visking tape against 2 liters of distilled water for 72 hr with three changes of water. Concentration of medium and ultrafiltration studies were done using a model 401 Amicon ultrafiltration cell with a UM-20 filter (solute cutoff 20,000 mol wt). After dialysis medium was dried by pervaporation in the dialysis bag and reconstituted to its original volume with fresh uncirculated medium. Medium concentrated by ultrafiltration was reconstituted in the same way.

Results. The morphologic appearance of colonies and colony cells was similar to that previously described for serum-stimulated colony formation (2). Initially colonies were composed of large mononuclear cells which had the appearance of immature myelocyte forms. By days 7–10 of culture, colonies were composed almost exclusively of mature granulocyte forms. Culture beyond this time led to the appearance in colonies of increasing numbers of mononuclear cells with small eccentrically placed nuclei.

Figure 1 shows the bone marrow colony-stimulating activity (CSA) effect of medium used to perfuse an isolated whole porcine spleen. The uncirculated control medium with 10% porcine serum had mild CSA. The levels of CSA in the medium rose dramatically during perfusion, reaching a peak at 4–12 hr, after which there was a marked fall. By 24 hr CSA could no longer be detected.

A similar but more profound rise of CSA is seen in Fig. 2 which illustrates the effect of medium used to perfuse a whole isolated porcine liver. The rise in CSA was more prolonged as well, but like that found in medium used to perfuse spleens, gradually decreased and after 42 hr CSA could no longer be demonstrated.

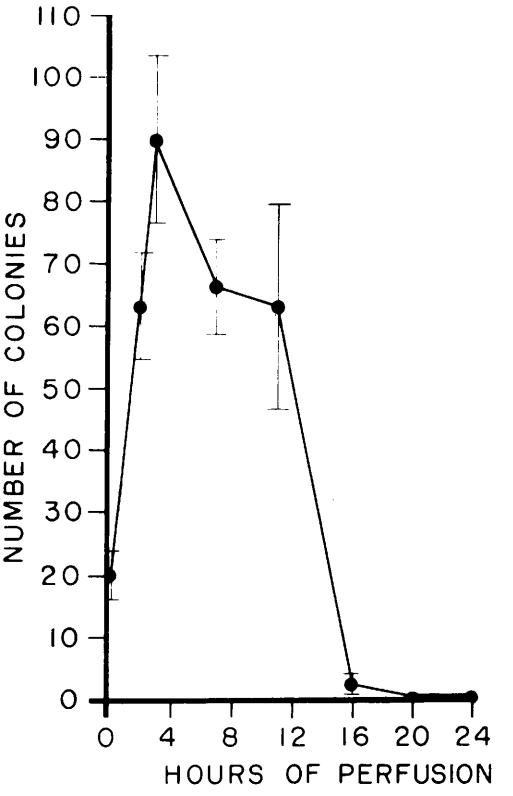

FIG. 1. Colony-stimulating activity of medium used to perfuse an intact porcine spleen at intervals from 0 to 24 hr. Control values for uncirculated medium are noted at zero time. Points represent mean number of colonies for 5 plates and bars represent SD.

To determine the nature of the factor in the perfusing medium the studies in Table I were performed. As shown, CSA was retained after heating, dialysis, and ultrafiltration. These studies indicate that the factor responsible for CSA in perfusate medium is a relatively heat stable material with a molecular weight greater than 20,000.

Some loss of CSA was noted after all treatment shown in Table I. Recovery of activity was however greater than 50% in all cases. The loss noted may represent alteration of the primary factor during these procedures, loss of accessory factors or most

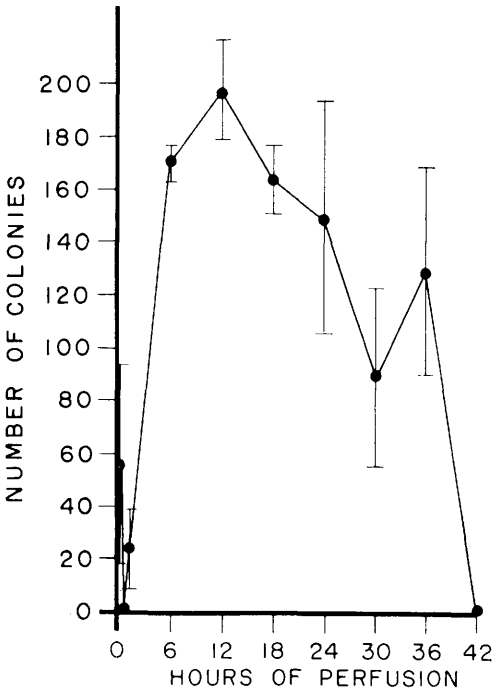

FIG. 2. Colony-stimulating activity of medium used to perfuse an intact porcine liver at intervals 0–42 hr. Control values for uncirculated medium are noted at zero time. Points represent mean number of colonies for 5 plates and bars represent SD.

likely technical difficulties.

Discussion. These studies demonstrated that bone marrow colony stimulating activity can be recovered from medium used to perfuse whole isolated liver and spleens. This finding adds to the body of knowledge already available that such activity can be recovered from many cell systems and body fluids.

TABLE I. The Effect of Heat, Dialysis and Ultrafiltration on CSA in Medium Used to Perfuse Porcine Liver for 24 hr.

Treatment	No. of colonies
Control medium (uncirculated)	0[a]
Perfusate medium	105 ± 9
Perfusate medium (heat 65°/30 min)	63 ± 9
Perfusate medium (dialyzed 72 hr)	70 ± 7
Perfusate medium (ultrafiltered)[b]	66 ±10

[a] Mean colony number of 4 plates with SD.

[b] Amicon ultrafiltration cell using UM-20 filter (solute cutoff 20,000 mol wt).

The significance of the rises and fall in CSA in the present studies has not been clarified. Cells from organs perfused in this system have been shown by dye exclusion tests to remain viable for up to 7 days and capable of responding to antigenic stimulation with sheep red blood cells (13). With increasing time of perfusion there is however increasing cell death. The data presented here suggest that CSA may come from some preformed cellular product released as cells die in the system.

Previous studies by Bradley (4) showed that "conditioned medium" prepared from mouse spleen and liver has little or no CSA. In these studies cell suspensions rather than intact organs were used and the time of incubation of the cells with medium was much longer—6 days. CSA during interval times is not reported for these cell sources. The finding of no CSA at day 6 of incubation might be related to the fall and disappearance of CSA in the present studies after prolonged perfusion.

There are definite physical similarities between the factor described here and that from conditioned medium, serum, and urine (4, 9, 10). All are nondialyzable, relatively heat stable, and of apparent similar molecular size. However, the properties described for all are crude and only further analysis will determine whether they are the same.

Summary. Bone marrow colony-stimulating activity was demonstrated in tissue culture medium used to perfuse isolated whole porcine livers and spleens. The factor responsible for this effect would appear to be similar to that previously described from serum, urine, and conditioned medium.

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