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Stimulation of Granulopoiesis After Removal of Cells Released by Newborn Mouse Spleen in Organ Culture[1] (38529)

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(Introduced by W. A. Robinson)

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Evidence that cells are released from normal (4, 11) or virus-induced leukemic (3) hematopoietic tissues in organ culture has been repeatedly reported.

Over the past few years, a technique which allows a more accurate cytological study of these "free" cells has been developed (6). This method involves the recovery of cells egressing from hematopoietic explants on a protecting silicone membrane since hemic cells in contact with these membranes retain their morphological differentiation for prolonged periods of time (8).

Studies presented here have shown that in the cell populations collected from newborn mouse spleen explants under these conditions, large numbers of granulocytic cells are present, in particular, during the early period of incubation. In addition, these experiments indicate that the removal of these free cells by collecting them on silicone membranes stimulates long-term granulopoiesis in these cultures.

Materials and Methods. Explants. Spleens are removed from BALC/c newborn mice less than 24 hr of age after the animals have been sacrificed by ether anesthesia and explanted in toto after cleaning them from surrounding attached tissues.

Culture technique. The organ culture method in perfusable chambers has been described in detail earlier (7). Briefly explants are cultivated on a millipore filter (HA 450 nm) inserted between two nontoxic synthetic rubber (Pluribar[5]) gaskets forming the walls of the chamber which is completed by a glass bottom and lid. The whole is held together by a special metallic device. Two ml of medium are injected through the wall of the lower compartment, filling it up and moistening the millipore filter. Most explants are covered with a "blanket" of silicone membrane prepared as mentioned below. Cultures are incubated in an ordinary atmosphere at 37°.

Great care is taken during the entire incubation period to keep the atmosphere of the chamber fully humidified since previous experiments have shown this to be critical in maintaining the explant in reliable satisfactory conditions. This is provided by adding 0.2–0.5 ml of fresh medium every one or 2 days to the upper compartment of the chamber. The whole medium is changed every 3 or 4 days.

Culture medium. The culture medium used in these experiments is referred to as O.T.G. medium and has the following composition:

Eagles MEM with sodium bicarbonate[6]	100 ml
Glutamine (30 mg/litre solution)	1 ml
Glucose (30 g/100 ml solution)	2 ml

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Fetal calf serum	25 ml
Penicillin	50,000 μm
Streptomycin	2 mg

Fresh medium is prepared every 2 wk. Its pH is 7.3.

Silicone membranes. These were kindly prepared and provided to us by Dr. A. Sausse (Service de Recherches de la Société Rhône-Poulenc Vitry-s-Seine, 94, France). All details regarding their manipulation have been published elsewhere (6). However, membranes slightly thicker than those previously described were used in these experiments. No particular care was taken when placing them on the explants to make them adhere.

Appraisal of the cell input in each culture. In order to get comparable cultures, only spleens from mice belonging to the same litter are used in a given experimental series. Repeated cell counts on individual spleens from control litters, dispersed as cell suspensions in T.C. medium, have indicated that the spleens of BALB/c newborn mice less than 24 hr of age have an average nucleated cell count of $1.6 \times 10^6 \pm 3 \times 10^5$.

Cytological studies. Cells collected on the membrane are processed according to a slight modification of our previously described technique (6) with great improvement of their cytological quality. When removed from the cultures, the membranes carrying the free cells are dried rapidly in a hair drier, fixed 10 min in methanol, and floated, cell down, on equal parts of Wright–Stain and distilled water at pH 7 for 6 min. The membranes are then transferred and floated on three drops Giemsa/2 ml distilled water at pH 7 for 20 min and rinsed in tap water. After being placed cells down on a drop of water on a glass slide, the membranes are allowed to dry overnight after which they are removed, and the stained cells are left on the glass slide. The slides are mounted with EUKITT and a coverslip.

Imprints from explants are performed on duplicate glass slides serially so as to get information on the cell populations in superficial and deeper areas of the explants. Once air dried they are stained with Wright Giemsa or for peroxidase.

Experimental protocol. In the present studies, all cultures have been incubated for a period of 21 days. All explants were covered with a silicone membrane immediately after introducing the explants in the chambers. Control cultures were left covered until the end of the incubation period (21 days).

In experimental (treated) cultures, the membrane was removed but not replaced at various periods of time. This study includes 10 experimental series of five to nine cultures depending on the number of spleens (newborn mice) available in the donor litter. As far as possible, one or two cultures were treated after 1 hr, 4, 8, and 12 days respectively, and at least one culture kept as an untreated control.

Evaluation of granulopoiesis enhancement in treated cultures. Granulopoiesis stimulation in cultures treated by free cell removal at a given period of time was estimated as follows: All imprints derived from each explant were read by two observers and the cultures classified according to the criteria summarized in Results after agreement of both observers. The data from all 10 experimental series were then put together and the relative number of cultures with granulopoietic activity matched for each time of treatment as well as for control cultures.

Results. 1. *Cells collected on the silicone membrane.* The cells recovered on the silicone membrane after 1 hr of incubation form an almost homogenous population made of late granulocytic cells including metamyelocytes and segmented polymorphonuclears (PMN), the former being predominant (Fig. 1). Beside these elements, cell debris and a very few red and lymphoid cells may be found.

By 3–4 days, coexisting with fibroblasts and reticular cells, representatives from erythroid, lymphoid and granulocytic cell line may occur on the membrane. Granulocytic cells are often arranged in large areas along the edges, others being scattered or grouped in islets in the center of the membrane.

On day 8 of incubation, a typical mixed cell population becomes apparent with presence of still large peripheral areas of granulocytic elements, mainly segmented PMN, whereas in the center granulocytic islets are scattered among small numbers of fibro-

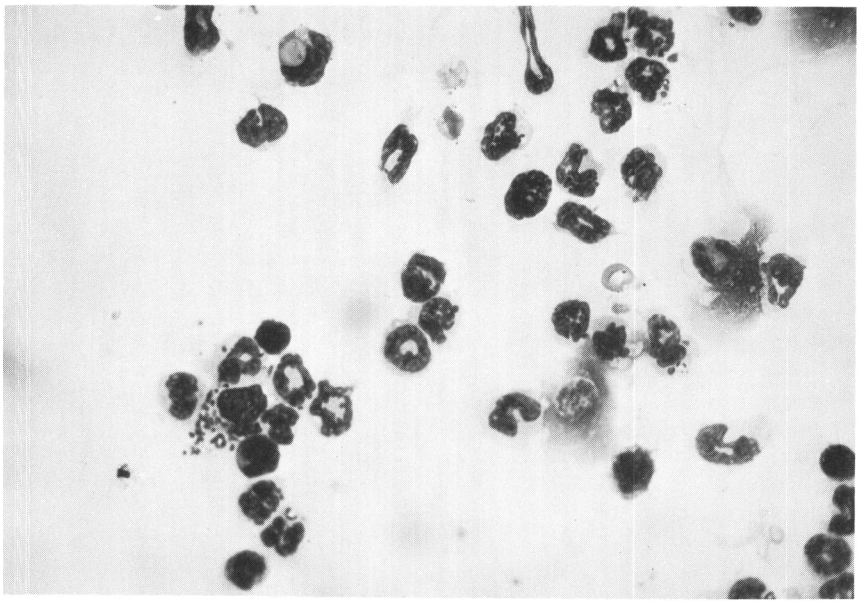

FIG. 1. Free cells recovered on silicone membranes from newborn mouse spleen organ cultures (Wright–Giemsa stain). One hour incubation: The large majority of the cells are granulocytic cells including metamyelocytes and segmented polymorphonuclears (PMN). × 700.

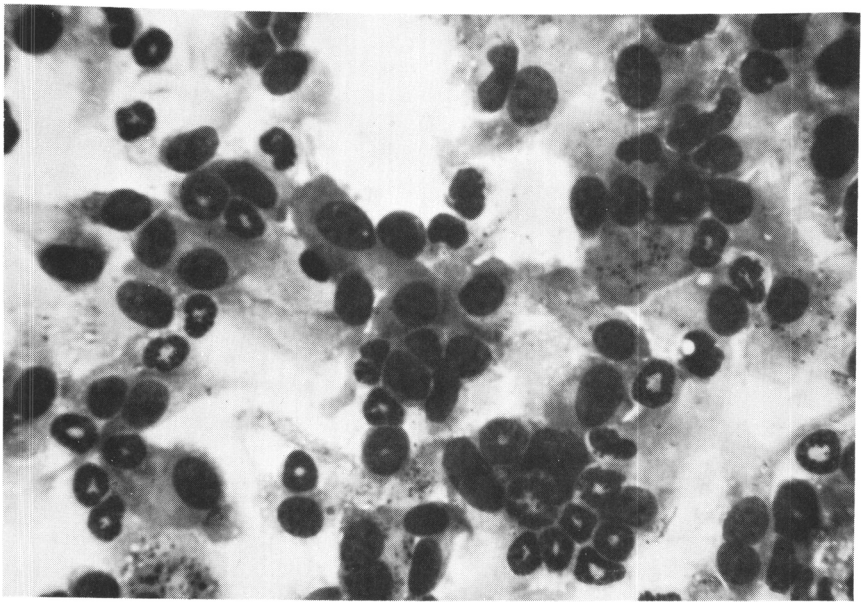

FIG. 2. Cells collected on silicone membrane on day 8 of incubation. Mixed population of granulocytic and mononucleated cells. × 700.

blasts and mainly, large mononuclear cells with an eccentrically placed nucleus (Fig. 2).

By day 12, the latter category of cells becomes predominant. Their morphology indicated above should be underlined because of their similarity to the so-called "monocytic" or macrophage cells described in late stages of hematopoietic cell cultures in semisolid media (14).

Finally, cells recovered from control cul-

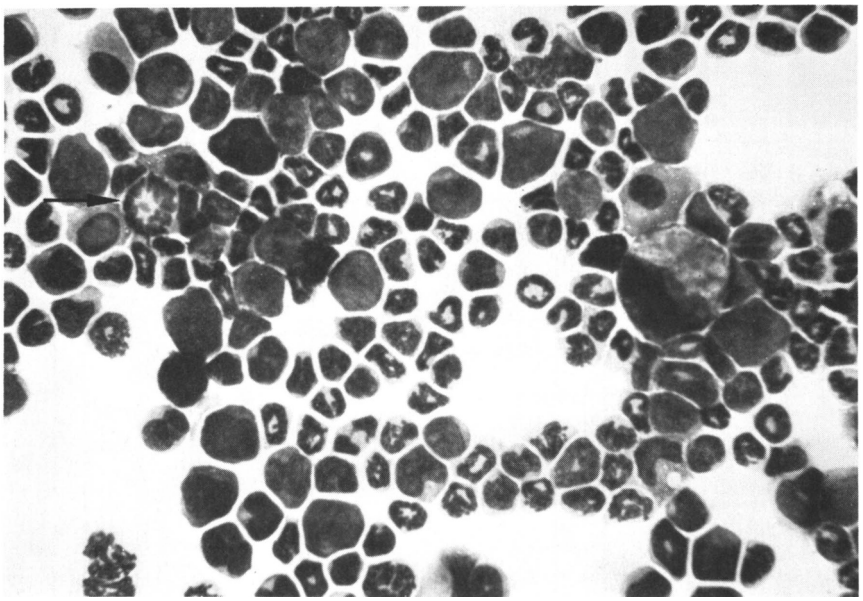

FIG. 3. Imprint from a 21-day old explant of new born mouse spleen in organ culture with myeloid cells at different stages of maturation and granulopoiesis, extended to the whole culture with reticular cells practically absent. One cell in mitosis is seen on the upper left of this field (arrow). × 700.

tures, i.e. on day 21 mainly include fibroblasts and dark prepyknotic mononucleated cells. The remainder appear to be degenerating forms derived from healthy mononuclear cells found on day 8 or 12 whereas the fibroblasts mentioned above could represent parts of the spleen capsule in close contact with the silicone membrane.

2. *Assessments of granulopoietic activity in individual cultures.* As mentioned in Methods, cultures could be divided into granulopoietic (Group A) and nongranulopoietic (Group B).

Cultures in group A (granulopoietic) showed on imprints the presence of myeloid cells at all stages of maturation including mature granulocytes (PMN). In some cultures myelopoiesis occupied more than 90% of the whole explant (Fig. 3). In others, granulopoietic activity was limited to one large area or to several smaller foci coexisting with various amounts of reticular cells. True granulopoiesis occurring in these explants was substantiated by (a) The presence of granulocytic cells at all stages of maturation: (b) the occurrence of PMN in 3-wk old cultures implying these cells must have matured *in vitro* based on their known short life span *in vivo*; (c) large numbers of mitotic figures suggesting that on day 21 early precursors are still replicating in this system.

Cultures were considered as nongranulopoietic when less than 10 granulocytes were found by scanning inspection of all the imprints obtained from the explant. However, nine of the treated cultures classified as nongranulopoietic showed, in addition to these few granulocytes, at least one or several foci of numerous mononuclear cells identical to those described in agar colonies from bone marrow cells (Fig. 4). It has been inferred that some of these mononuclear cells may be derived from myeloid cells (14).

3. *Effect of free cell removal on granulopoiesis in 21 day old culture.* From our preliminary data presented in this paper (Table I) no significant time effect of cell removal on long term granulopoiesis could be substantiated. However, when results from all times of treatment were pooled together, it appeared that in treated cultures there were almost three times as many granulopoietic explants as in control cultures, and statistical analysis indicated this difference was significant (chi-square: 4.42 on one degree of freedom: $P < 0.01$.

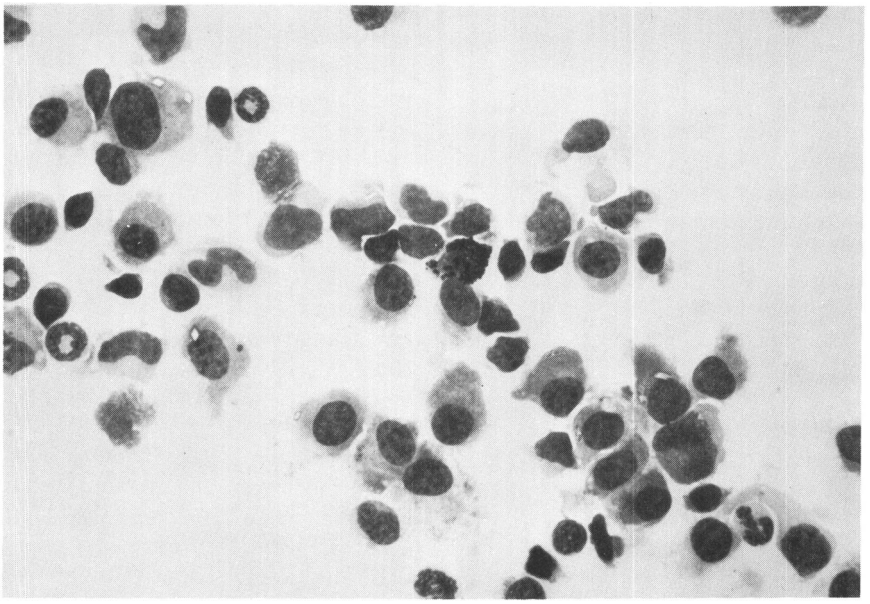

FIG. 4. Few granulocytic cells in an islet of mononucleated cells similar to those described as macrophages in colonies from hemopoietic cell cultures in agar. × 700.

It may be pointed out that preliminary studies have shown that decreased granulopoiesis in control cultures left in contact with the silicone membrane till the end of the incubation period is not related to some toxicity of the silicone itself, since when explants are cultured in the absence of a protecting silicone membrane, this also results in the loss of granulopoietic activity after 15–20 days of incubation. Moreover, the silicone membrane technique has been applied to long term (4–5 wk) cultures of mouse and guinea pig spleen fragments with improvement of our results as far as the maintenance of the lymphoid cell line was concerned (Goube de Laforest and Voisin, unpublished results).

TABLE I. DISTRIBUTION OF 21-DAY OLD CULTURES WITH RESPECT TO THE TIME FREE CELLS HAVE BEEN REMOVED (TREATMENT).

Time treatment was applied	Total cultures treated at the same time	Group A "granulopoietic" Number	Group A "granulopoietic" %	Group B non-granulopoietic
1 hr	10	6	60	4
Day 4	13	5	38	8
Day 8	12	9	74	3
Day 12	9	5	56	4
Total treated	44	25	57	19
Untreated controls	10	2	20	8

Discussion. These experiments indicate that the removal of free cells released by newborn mouse spleen explants stimulates long term granulopoiesis in the culture system used here provided this treatment is applied at appropriate periods of incubation. Similar data have been reported as early as 20 yr ago by E. E. Osgood and his group (16), based on bone marrow cell cultures carried out by the gradient culture technique. These authors found that when bone marrow cell suspensions were allowed to settle on a 45° angle inclined glass slide placed on the bottom of a culture bottle, in addition to the gradient in oxygen tension, oxidation-reduction potential and number of cells per unit volume of medium from above downward to the depth in this culture system, "there is an opportunity for the more motile, mature cells to migrate over the edge of the slide." Since removing and changing the glass slide proved to give better cell growth than simply inclining the culture bottles themselves, it became clear to Osgood and coll that re-

moval of the mature cells "was an important factor in the success of the method." This was confirmed by further studies, allowing immature cells to affix themselves to the slide while the more mature elements were removed by gentle rocking of the bottle to place them in suspension. Osgood has concluded from these studies that the chemically mature cells in both granulocyte, lymphocyte and monocyte series produces a relatively unstable inhibitor of cell division postulating humoral mechanism of homeostasis for hemic—and in fact other cells which differentiate.

Hematopoietic activity *in vivo* involves: (a) Differentiation and self-renewal of a stem cell pool, (b) maturation of the differentiated cells, (c) release of mature cells and their removal by the blood stream.

Even though the first two steps can be more or less achieved *in vitro*, in most culture techniques, mature cells fail to be removed by any circulatory drainage. It could be questioned whether or not these accumulating mature cells may be involved in negative feedback mechanisms limiting further maintenance of hematopoietic activity.

Our own results in agreement with those from other groups (1), (17), (20), support this concept. However they do not allow to anticipate whether the *replication* and/or the *differentiation* of granulocyte precursors are indeed controlled by such a mechanism. Although the definite cell type responsible for the inhibition of granulopoiesis in our culture system has not so far been established, it may be pointed out that most of the free cells collected under our culture conditions are granulocytes and, later, mononucleated cells which also represent end forms in the development of granulocytic–macrophage colonies in the agar-culture system (14). Whether these cells may exert a negative feed-back on their own production remains at the present time a matter of controversy. In fact, increasing evidence strongly implicates both macrophage–monocytes (2, 5, 9, 15) and granulocytes (10, 19), in the production of the colony stimulating factor (CSF) required to stimulate granulocytic colony differentiation and maturation *in vitro*. On the other hand, it has been suggested that granulocytes may inhibit their own production in both *in vivo* cultures (1), liquid cultures (20), and agar cultures (17) of bone marrow. The discrepancy between this data may, however, only be apparent and reflect differences amongst animal species or result from different approaches to the feedback control of granulopoiesis by mature granulocytic cells. However, the possibility that normal healthy granulocytes in excess exert a negative feedback influence on granulopoiesis, and their breakdown contributes in maintaining a steady state of myeloid activity should also be taken into consideration. That both stimulatory and inhibitory controls of their own production might be carried out by mature granulocytes has been recently suggested (12, 21).

Finally, the fact that all our cultures in this study were made without any addition of known CSF should be pointed out since, in agar cultures, hematopoietic cells are unable to differentiate and mature in the absence of this factor (14). This suggests that, if CSF is a universally needed factor for granulopoiesis, this factor is either brought by the explants themselves or synthesized de novo by CSF producing cells present in the spleen at the time it is explanted or which have differentiated *in vitro*.

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