Experimental analysis of cell interactions during hemopoiesis

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ABSTRACT The principal approach was to study hemopoiesis on different stromal cell underlayers (fibroblasts or fibroblast-like cells covering a foreign body implanted into the peritoneal cavity of mice or other rodents) after intraperitoneal transplantation of syngeneic, allogeneic, and xenogeneic hemopoietic cells. The data obtained are compared with the results of experiments on repopulation of ectopic hemopoietic territories (under the mouse kidney capsule) by syngeneic and xenogeneic hemopoietic cells. Competitive cell interactions are described that occur during repopulation of the hemopoietic stroma or formation of the hemopoietic foci on cellulose acetate membranes (CAMs) in the peritoneal cavity of irradiated mice by genetically different hemopoietic cells transplanted to these animals (multicomponent radiation chimeras). The model of xenogeneic and multicomponent radiation chimeras was reproduced in long-term bone marrow cultures, where hemopoietic cells of different genotypes coexisted, without any competitive cell elimination. The second part of this review deals with hemopoiesis on stromal cell underlayers, formed by cells of different origin, different stages of development, and obtained from other sources. These underlayers were formed on CAMs *in vitro* and then transferred into the peritoneal cavity of irradiated mice, which subsequently received intraperitoneal injections of donor hemopoietic cells. Specific features of hemopoiesis on stromal underlayers formed by the following cell types are described: (1) fibroblasts from mouse embryos at different developmental stages; (2) fibroblasts from the skin, liver, and bone marrow of 17-day mouse fetuses and newborn mice; (3) fibroblasts from the monolayer cultures of mouse and rat bone marrow; (4) 3T3 cell line; (5) hepatocytes of 17-day mouse fetuses or sexually mature rats; (6) newborn mouse kidney cells; and (7) cells transgenic for the erythropoietin gene. The phenomena observed in these experiments and their probable mechanisms are discussed.

KEY WORDS: *hemopoiesis, cell interactions, hemopoietic cell differentiation, stromal cells, experimental models*

Introduction

Normal hemopoiesis is maintained due to complex interactions between the hemopoietic cells, stromal cells (an important component of hemopoietic microenvironment), and growth factors.

During embryonic development, hemopoiesis is transferred from one organ to another: from the yolk sac into the liver, spleen, and eventually into the bone marrow. In terms of phylogeny, hemopoiesis becomes gradually concentrated on certain territories with an appropriate stroma, thus leading to the formation and specialization of hemopoietic organs: the granulocyte lineage prevails in the bone marrow and erythrocyte lineage in the spleen. These and other well-known facts suggest a regulatory role of the stroma in differentiation of hemopoietic cells.

Numerous recent experimental data clearly showed that close associations or direct interactions between the hemopoietic and stromal cells are essential for hemopoiesis and that the stromal cells can release or accumulate hemopoietic growth factors (Gimble *et al.*, 1989; Abboud and Pinzani 1991; Izumi-Hisha *et al.*, 1991; Paul *et al.*, 1991; Verfaillie *et al.*, 1994; Davis *et al.*, 1995; Gibson *et al.*, 1995). Moreover, the growth factors associated with the stromal cell surface exerted a greater effect on the stem cells and other clonogenic hemopoietic cells than soluble factors (Otsuka *et al.*, 1991; Sutherland *et al.*, 1991; Toksoz *et al.*, 1992; Gibson *et al.*, 1995).

Abbreviations used in this paper: CFU-s, colony-forming unit in spleen; BFU-e, burst-forming unit erythroid; CFU-e, colony-forming unit erythroid; CFU-gm, colony-forming unit granulocyte-macrophage; GM-CSF, granulocyte-macrophage colony stimulating factor; Epo, erythropoietin; EpoR, erythropoietin receptor; rHuEpo, recombinant human erythropoietin; IL-3, interleukin-3; LPS, lypopolysaccharide; CAM, cellulose acetate membrane; LTBMC, long-term bone marrow culture; SCF, stem cell factor.

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Fig. 1. Cross section of granulocytic colony and fibroplast (fibrolast-like cell) underlayer of the surface of CAM in the peritoneal cavity of the irradiated mouse. *(Magnification 15000x).*

Today, various experimental models and methodological approaches are used to analyze interactions between hemopoietic and stromal cells, roles of individual growth factors and their combined effects, significance of different stromal cell components for hemopoietic cell differentiation, role of extracellular matrix in cell interactions, etc. (Gimble *et al.*, 1990; Knospe *et al.*, 1990; Rios and Williams, 1990; Arkin *et al.*, 1991; Clarke and McCann, 1991; Izumi-Hisha *et al.*, 1991; Paul *et al.*, 1991; Aizawa *et al.*, 1992, 1994; Coulombel *et al.*, 1992; Fukumoto, 1992; Moore, 1992; Siczkowski *et al.*, 1992; Toksoz *et al.*, 1992; Deeg and Huss, 1993; Liesveld *et al.*, 1993; Rogers and Berman, 1993; Funk *et al.*, 1994; Kollman *et al.*, 1994; Kubanek, 1994; Plum *et al.*, 1994; Verfaillie *et al.*, 1994; Ye *et al.*, 1994; Davis *et al.*, 1995; Gibson *et al.*, 1995; Nishijima *et al.*, 1995; Sakai *et al.*, 1995; Stacchini *et al.*, 1995; Almeida-Porada *et al.*, 1996; Wineman *et al.*, 1996; etc.).

These problems have also been addressed in our research. Our purpose was to sequentially alter each of the three main hemopoietic components, i.e., stromal cells, hemopoietic cells, and hemopoietic growth factors, and to register and analyze the resulting changes in hemopoietic cell proliferation and differentiation. To this end, we performed experiments with: (1) different types of stromal cell underlayers; (2) different hemopoietic cells, including bone marrow cells of normal mice or other donor species, human erythroleukemia K-532 or myeloleukemia HL-60 cells; and (3) changes in the growth factor environment resulting from X-irradiation, bleeding, use of stromal cells transgenic for the growth factor genes, etc. In each experimental variant, changes in hemopoiesis (proliferation and differentiation of hemopoietic cells) were registered.

Experimental procedures

The experimental models used included radiation chimeras, long-term bone marrow cultures, and ectopic bone marrow transplantation. The basic model, however, was the formation of hemopoietic foci on the surface of foreign bodies implanted into the peritoneal cavity of experimental animals (Seki, 1973).

We implanted 10x20 mm pieces of cellulose acetate membrane for electrophoresis (CAM), Millipore filter, glass slide, etc., into the peritoneal cavity of recipient mice or other animals, thus inducing aseptic inflammation characterized by a sequence of specific cell stages (leukocyte, macrophage, and fibroblast stages). After five to ten days, the surface of the implants became covered with a cell sheet. Our electron-microscopic studies showed that the latter consisted of three to ten layers of fibroblast-like cells and collagen fibers (Michurina *et al.*, 1980; Khrushchov and Michurina, 1987), (Fig. 1).

Our previous experiments with xenogeneic radiation chimeras confirmed the bone marrow (donor) origin of these fibroblast-like cells (Michurina *et al.*, 1991a). These chimeras, obtained by transplanting the rat bone marrow cells to lethally irradiated mice, received intraperitoneal implants (glass slides) when all proliferating bone marrow cells had the rat (donor) karyotype (Fig. 2). Analysis of proliferating cells on the surface of glass slides, performed five to eight days after implantation, showed that all these cells had the rat karyotype as well. Their ultrastructural features during mitosis and at the other phases suggest that these were fibroblast-like cells (fibroblasts) (Fig. 3). Autoradiographic experiments, Southern blotting, and *in situ* hybridization with the ID sequence of rat DNA, a specific marker of the rat genome (Khrushchov *et al.*, 1988a; Khrushthov, 1992; Michurina *et al.*, 1991b,1992) (Fig. 4) also confirmed the donor origin of cells covering the surface of a foreign body implanted into the peritoneal cavity of radiation chimeras.

When the fibroblast-like cell layer developed on the surface of the implant (CAM), experimental animals were X-irradiated at

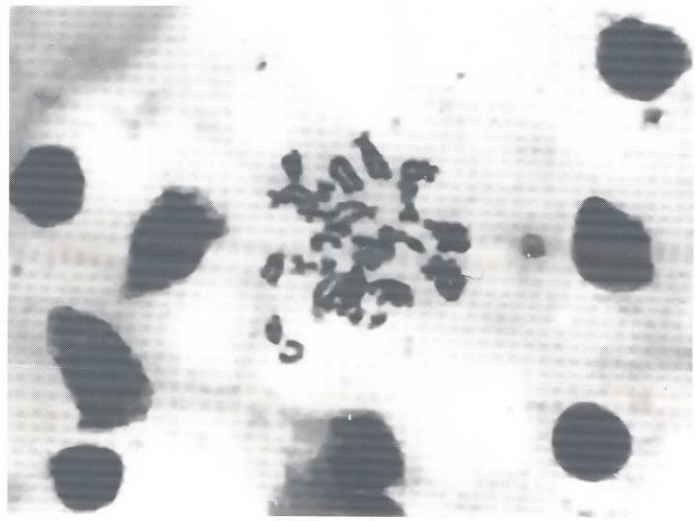


Fig. 2. Dividing rat (donor) cell on the surface of the foreign body from the peritoneal cavity of xenogeneic (rat in mouse) radiation chimera.

doses of 6.1-6.6 Gy and then received intraperitoneal injection of a suspension of the donor bone marrow cells. Under the standard conditions, $1x10^6$ nucleate cells in 1 ml of the culture medium were injected. If allo- or xenogeneic donors were used, the number of injected cells was increased to $(10-40)x10^6$.

Seven to ten days after hemopoietic cell transplantation, visual and microscopic analysis could reveal hemopoietic cell foci (colonies), mainly of the granulocyte type, on the CAM surface.

This approach, allowed us to develop experimental models for analysis of cell interactions during hemopoiesis by sequentially changing different components of the system under study.

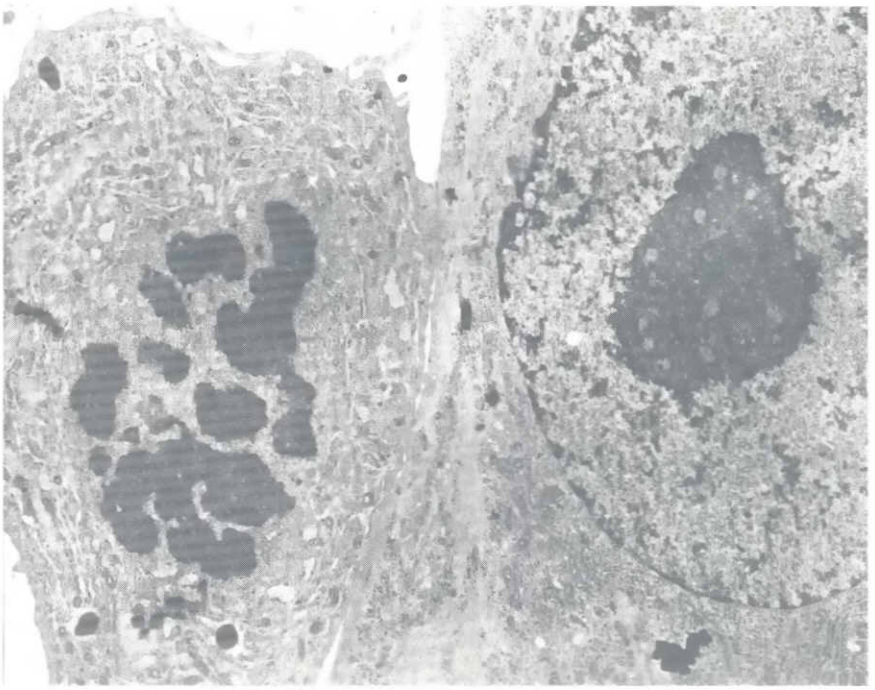


Fig. 3. Ultrastructure of the dividing and interphase fibroplasts (fibroplast-like cells) on the surface of a foreign body five days after implantation in the peritoneal cavity of a xenogeneic (rat in mouse) radiation chimera. *(Magnification 12000x).*

Transferring CAMs covered by cell underlayers from the subcutaneous connective tissue into the peritoneal cavity and vice versa

To evaluate the stromal role of cells of the underlayer, experiments on CAMs crosstransfer were performed. In one group of mice [(CBAxC57Bl)F1 females weighing 22-24g], CAMs were implanted under the dorsal skin, and in another group, into the peritoneal cavity. Seven days after the operation, all mice were X-irradiated, and CAMs were transferred from the subcutaneous connective tissue into the peritoneal cavity of secondary recipients and *vice versa*. The secondary recipients were irradiated three to five days prior to CAMs crosstransfer to prevent their fibroblast precursors from contributing to the formation of underlayers on CAMs. Bone marrow cells were injected into secondary recipients at the site of CAM implantation (Fig. 5). Seven days after the injection, CAMs were removed and examined. The number and size of hemopoietic cell colonies on the fibroblast underlayer formed in the peritoneal cavity and transferred subcutaneously were similar to those developing in the peritoneal cavity under the standard conditions. The underlayer formed in the subcutaneous connective tissue and transferred to the peritoneal cavity did not support the formation of hemopoietic colonies (Khrushchov and Michurina, 1987; Michurina *et al.*, 1991b). Granulocyte clusters occasionally observed on such transplants were dissimilar to the colonies formed on the peritoneal underlayer in terms of their size or quantity. Cell composition and structure of the underlayers developing in the peritoneal cavity and under the skin were morphologically similar. The differences between fibroblast-like cells of the underlayers formed on CAMs in the peritoneal cavity and subcutaneous connective tissue of rat-in-mouse radiation chimeras concerned their reaction with a specific antiserum to rat bone marrow cells (Khrushchov *et al.*, 1977,1981,1984a,b; Vasilieva *et al.*, 1977,1978; Khrushchov, 1981; Mikhina *et al.*, 1981; Khrushchov and Michurina,1987; Michurina *et al.*, 1992; Figs. 6A,B,C). Immunohistochemical analysis of light- and electron-microscopic preparations showed that this antiserum bound to fibroblast-like cells covering a glass slide implanted into the subcutaneous connective tissue (Figs. 7A,B) but did not bind to such cells from the peritoneal cavity of radiation chimeras (Khrushchov and Michurina, 1987; Khrushchov, 1992; Michurina *et al.*, 1992). These results suggest that the cells forming these two types of underlayers may belong to different fibroblast populations (Khrushchov, 1974, 1976, 1989, 1992; Voskresenskiy *et al.*, 1988). According to Rios and Williams (1990), fibroblast-like cell lines derived from the adult mouse skin do not maintain hemopoiesis *in vitro*, in contrast to stromal cells of the adult bone marrow.

Hemopoietic colonies formed on CAMs in the peritoneal cavity and mesentery of mice after transplantation of xenogeneic bone marrow cells

The ability of the peritoneal mouse fibroblast underlayers to support the development of hemopoietic colonies from the transplanted bone marrow cells of other rodent species was analyzed in the following experiments. Seven days after implanting CAMs in the peritoneal cavity, the recipient mice were irradiated and received intraperitoneal injections of xenogeneic bone marrow cells ($5-10x10^6$ cells each) obtained from Wistar rats, golden or dwarf hamsters, or Guinea pigs. Seven days later, rat or golden hamster bone marrow cells formed hemopoietic (granulocyte) colonies on the mouse cell underlayer. The donor origin of these colonies was confirmed by their positive reaction for alkaline phosphatase, karyotyping, and binding of specific antisera against the rat or golden hamster bone marrow cells. No such colonies developed on the mouse cell underlayer if the dwarf hamster or Guinea pig bone marrow cells were transplanted.

After the intraperitoneal transplantation of either syngeneic or rat and golden hamster bone marrow cells, we also observed formation of hemopoietic colonies in the mesentery of the recipi-

ent mice (Figs. 8A,B,C). As in the previous case, the dwarf hamster or Guinea pig bone marrow cells formed no such colonies.

Therefore, xenogeneic bone marrow transplantation is successful in the same experimental variants as those used for producing xenogeneic radiation chimeras: repopulation of the hemopoietic organs of irradiated mice after intravenous transplantation of the xenogeneic bone marrow cells is achieved only if rats or golden hamsters, rather than dwarf hamsters or Guinea pigs, are used as donors. These results suggest that hemopoiesis requires a certain conformity between the hemopoietic cells, on the one hand, and stromal cells and growth factors, on the other. The data obtained in our experiments on ectopic transplantation of the syngeneic and xenogeneic bone marrow confirm this conclusion.

Ectopic transplantation of syngeneic and xenogeneic bone marrow to irradiated mice

It is known that a fragment of syngeneic bone marrow (not reduced to cell suspension) transplanted under the kidney capsule of an irradiated mouse forms a hemopoietic focus. This is preceded by the formation and resorption of spongy bone and development of stroma in which hemopoietic cells subsequently proliferate and differentiate.

In our experiments, fragments of the mouse and dwarf hamster bone marrow were simultaneously transplanted under the kidney capsule of an irradiated mouse at a certain distance from one another. Seven to ten days after the operation, both transplants demonstrated active hemopoiesis. Tests for alkaline phosphatase (positive for the dwarf hamster granulocytes and negative for the mouse granulocytes) and binding of specific antibodies against the mouse and dwarf hamster bone marrow cells showed that the mouse bone marrow transplant contains mouse hemopoietic cells and the dwarf hamster bone marrow transplant contains dwarf hamster hemopoietic cells. During this period, the kidney region with the transplants was locally irradiated at a dose of 7.4 Gy, and two to three hours later the recipient mice received intravenous injections of the donor bone marrow cell suspension. One group of mice received mouse bone marrow cells and another group-dwarf hamster bone marrow cells ($40x10^6$ cells each). The third group was not injected and served as the control. The extramedullary transplants were analyzed five to seven days after injection. In mice receiving mouse bone marrow cells, small hemopoietic cell foci (colonies) developed only in the transplant stroma. In mice receiving dwarf hamster bone marrow cells, such colonies developed only in the dwarf hamster transplant stroma, whereas the mouse transplant stroma was depleted by irradiation and showed no signs of hemopoiesis (Fig. 9). In the control mice, both transplants consisted of empty stroma.

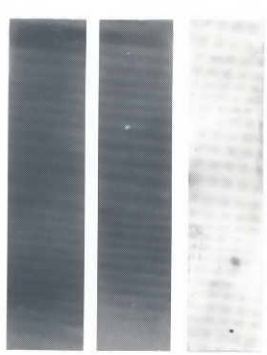


Fig. 4. Southern blot hybridization analysis of DNA from cells covering a foreign body (coverslip) in peritoneal cavity: *of rat (**1**), rat-mouse radiation chimera (**2**) and mouse (**3**) with ^{32}P-labeled ID-sequence of rat.*

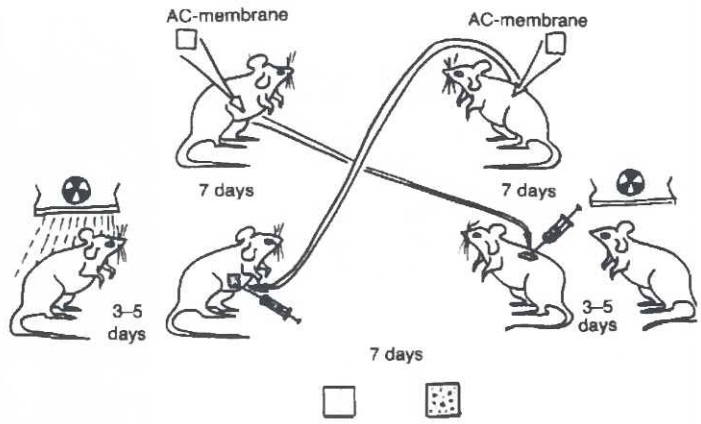


Fig. 5. Scheme of experiment with cross-transfer of CAM's with a cell underlayer formed in subcutaneous connective tissue into peritoneal cavity of mice and *vice versa*.

Formation of hemopoietic colonies on CAMs in the peritoneal cavity or mesentery of some rodents after intraperitoneal transplantation of syngeneic, allogeneic, and xenogeneic bone marrow

In the next experimental series, several other rodents, in addition to mice, served as recipients. These were rats, golden and dwarf hamsters, and guinea pigs. Seven days after the intraperitoneal CAM implantation, they were irradiated at doses specifically selected to suppress hemopoiesis and immunological response and to deplete hemopoietic organs without causing the intestinal syndrome. Recipients of every species were subdivided into groups (10-15 animals each) and, 18-20 h after irradiation, each group received an intraperitoneal injection of $10x10^6$ bone marrow cells from one of the following donor species: mouse, rat, golden or dwarf hamster, or guinea pig. Seven days later, CAMs were removed and examined. The results agreed with those obtained in the experiments described above. Thus, the mouse fibroblast underlayer supported the formation of hemopoietic foci by transplanted rat and golden hamster bone marrow cells, rather than by dwarf hamster or guinea pig cells. On the rat fibroblast underlayer, colonies developed from transplanted mouse, rat, or golden hamster bone marrow cells. The maximum number of colonies on the golden hamster cell underlayer was recorded after the allogeneic bone marrow transplantation, while few colonies developed after the rat bone marrow transplantation. The same was true for the dwarf hamster cell underlayer. The underlayer formed in the guinea pig peritoneal cavity supported formation of few hemopoietic colonies (10-20 per membrane) by from allogeneic bone marrow cells alone.

Studies on the formation of hemopoietic foci in the mesentery of the same rodents (after irradiation and intraperitoneal injection of syn-, allo-, and xenogeneic bone marrow cells) produced similar results.

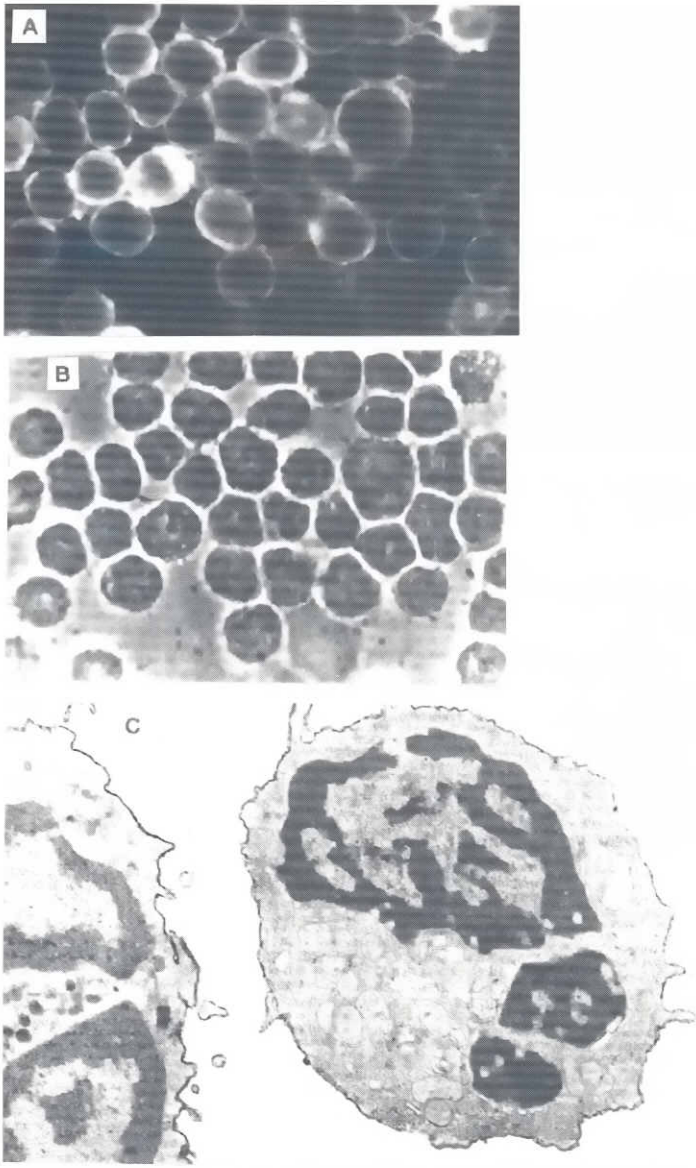


Fig. 6. Rat bone marrow cell monolayer. (**A**) *Indirect immunofluorescent method using specific antibodies against rat bone marrow cell membranes.* (**B**) *The same, phase contrast.* (**C**) *Indirect immunoperoxidase electron-microscopical method using specific antibodies against rat bone marrow cell membranes.*

Transplantation of syngeneic(xenogeneic bone marrow cell mixture to lethally irradiated mice. Competitive cell interactions. Long-term culture of syngeneic and xenogeneic bone marrow cells

The ability of stromal cells to support, in certain cases, proliferation and differentiation of xenogeneic hemopoietic cells was the reason for analyzing the behavior of syngeneic and xenogeneic bone marrow cells transplanted together. It was not known, whether syngeneic and xenogeneic (relative to the host stroma) hemopoietic cells can proliferate simultaneously, provided the immunological response is suppressed by irradiation; whether syngeneic cells have any advantages over the xenogeneic any; whether the stroma exerts any regulatory effect; and what probable mechanisms of interactions between the hemopoietic and stromal cells are. To elucidate these problems, we performed experiments with radiation mouse chimeras (Khrushchov *et al.*, 1988b), hemopoietic colony formation on CAMs implanted in the peritoneal cavity, and long-term bone marrow cultures (Khrushchov *et al.*, 1987).

Multicomponent radiation chimeras

Hybrid F_1(CBAxC57Bl) mice were irradiated at doses of 6.5-7.4 Gy and, one day later, received intravenous injections of bone marrow cell mixtures from two (mouse and rat) or three donors (mouse, rat, and golden hamster). The ratio of donor cells varied in different experiments. To determine the origin of hemopoietic cells in radiation chimeras, we made chromosome preparations of bone marrow cells; made tests for alkaline phosphatase with bone marrow, peripheral blood, and peritoneal exudate smears and with the spleen and liver sections; we performed indirect immunofluorescence analysis using species-specific antibodies against the membrane antigens of rat, mouse, and golden hamster bone marrow cells.

In one experiment, each irradiated mouse received a mixture of $1x10^6$ mouse, $25x10^6$ rat, and $25x10^6$ golden hamster cells. One week after the injection, the bone marrow of these mice contained proliferating cells of all three genotypes. Two weeks later, however, only proliferating mouse cells could be found on chromosome preparations. Three to four weeks after the injection, tests for binding of species-specific antibodies and for alkaline phosphatase revealed the presence of single rat and golden hamster cells or their small clusters in the spleen, liver, bone marrow, and peritoneal exudate of recipient mice.

A similar situation was observed with mice that received $40x10^6$ rat and $20x10^6$ mouse bone marrow cells.

Therefore, bone marrow cells from two or three donor species, injected as a mixture, can repopulate the hemopoietic organs of irradiated mice, thus producing radiation chimeras with mosaic (two- or three-component) hemopoietic tissue. After three weeks, however, predominant proliferation of syngeneic cells leads to displacement of xenogeneic cells from the bone marrow. At this time, xenogeneic cells are largely represented by their mature forms.

To analyze the process of xenogeneic cell displacement (substitution), we changed the ratio of syngeneic and xenogeneic bone marrow cells in the injected mixture. Seven to 12 days after the irradiated mice received $1x10^6$ mouse and $20x10^6$ rat bone marrow cells, 50-75% of proliferating cells in their bone marrow had the mouse karyotype and 25-50% the rat karyotype. In the spleen, rat and mouse hemopoietic cell colonies developed. By days 18-21 after irradiation, proliferating rat cells were no more found, and only mouse hemopoietic cells continued to proliferate. When the number of mouse cells in the mixture was reduced to $1x10^5$, the proportion of rat mitoses decreased from 90-97% on days 7-12 to 50% on day 16 and no cells with the rat karyotype were found on days 21-27.

These results suggest that xenogeneic (rat) cells, injected together with the syngeneic ones, repopulate the hemopoietic tissue and actively proliferate there but soon become displaced by the syngeneic cells. The time course of this process depends on the syngeneic to xenogeneic cell ratio: the less the amount of syngeneic cells in the injected mixture, the more abundant are proliferating xenogeneic cells and for longer periods of time they persist.

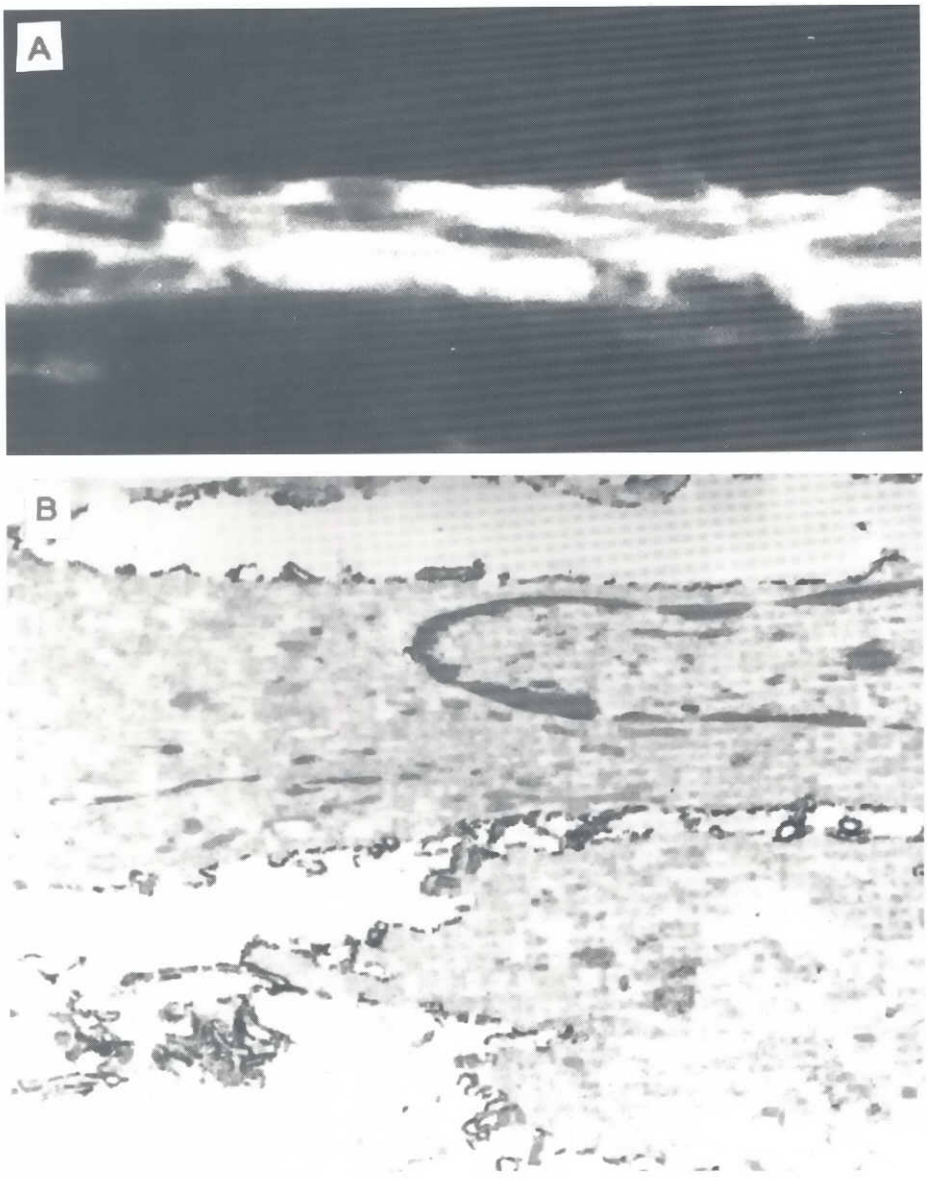


Fig. 7. Fibroplasts (fibroplast-like cells) on the foreign body in subcutaneous connective tissue of xenogeneic (rat in mouse) radiation chimera. (**A**) *Indirect immunofluorescent method with antibodies against rat bone marrow cell membranes.* (**B**) *Indirect immunoperoxidase electron-microscopical method with antibodies against rat bone marrow cell membranes.*

In other experiments, we compared the ability of rat and golden hamster bone marrow cells to repopulate the stroma of hemopoietic organs in irradiated mice. If these mice received $20x10^6$ bone marrow cells of each species, all mitotic cells detected on day 7 after irradiation or later had the rat karyotype. In addition, species-specific antibodies revealed the presence of single golden hamster cells. If $50x10^6$ golden hamster and $5x10^6$ rat cells were injected, mitotic cells in the recipient's bone marrow had either rat or golden hamster karyotype, rat cells getting prevalent by day 16 after irradiation.

Thus, with immune response suppressed by irradiation, genetically different donor hemopoietic cells compete for the host hemopoietic stroma. Mouse cells have the greatest potential for repopulating the host stroma (to which they are syngeneic), then follow rat cells and, finally, golden hamster cells.

Note that the time course of competitive displacement of «less potent» hemopoietic cells from the host stroma depends on the ratio of donor cells. An increased proportion of less potent cells in the injected mixture makes them more successful in primary competition for the stroma. However, the ratio of mitotic syngeneic and xenogeneic cells does not remain constant but rapidly changes in favor of syngeneic cells. In other words, hemopoietic stem cells perpetually compete for certain niches (domains) of the stromal microenvironment, rather than reside in them permanently, and more competitive cells are thus selected. We attribute the advantage of syngeneic cells in this competition to their more efficient use of growth factors, but other explanations are equally possible.

Hemopoietic foci formed on CAMs after transplanting a mixture of syngeneic and xenogeneic bone marrow cells

The competitive interactions between the donor hemopoietic cells of different phenotypes manifest themselves also during formation of hemopoietic foci on the fibroblast-like cell underlayers covering the intraperitoneal CAM implants. To identify hemopoietic cells giving rise to these foci, we used species-specific antibodies against the mouse, rat, and golden hamster bone marrow cells. All recipient mice received intraperitoneal injections of a mixture containing $1x10^6$ mouse, $1x10^7$ rat, and $1x10^7$ golden hamster bone marrow cells. Nine days later, the mean ratio of mouse, rat, and golden hamster hemopoietic cell colonies per membrane was 70:14:1; on day 20 after the injection, however, only mouse cell colonies could be found. In other experimental series, we transplanted bone marrow cells from two donor species in different proportions: $1x10^6$ mouse + $1x10^7$ rat or golden hamster cells or $1x10^7$ mouse + $1x10^6$ rat or golden hamster cells, and the results registered on days 7-9 after transplantation also provided evidence for competitive cell interactions.

The results described here agree with data obtained in experiments on producing multicomponent radiation chimeras. They suggest that competition for the stroma at the level of stem cells repopulating hemopoietic organs is also characteristic for the clonogenic hemopoietic cells committed to differentiation into granulocytes, which form hemopoietic colonies on CAMs.

Multicomponent long-term bone marrow cultures

The opposite results were obtained when bone marrow cell mixtures from two or three rodent species were cultivated according to Dexter on the mouse stromal cell underlayer.

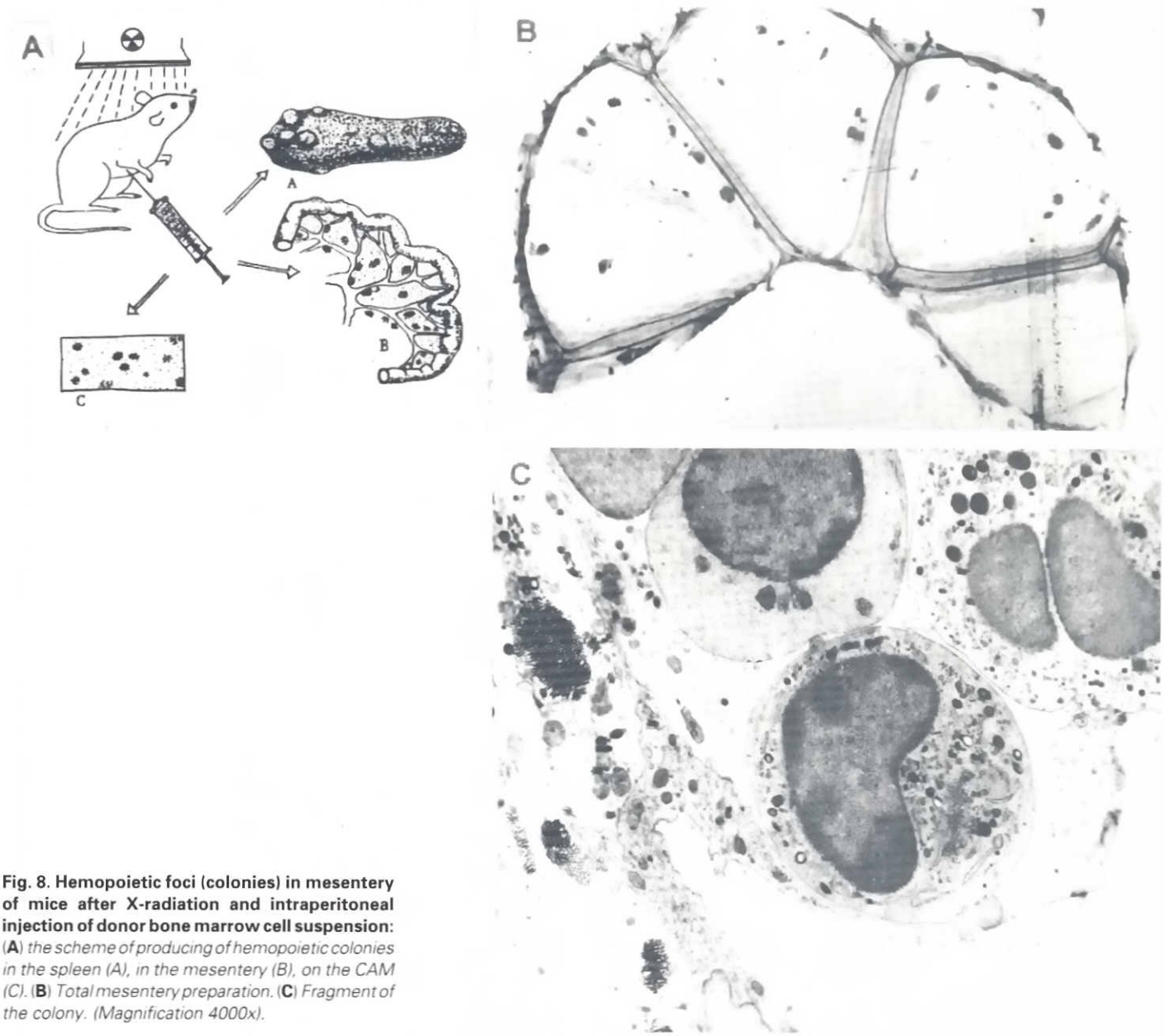


Fig. 8. Hemopoietic foci (colonies) in mesentery of mice after X-radiation and intraperitoneal injection of donor bone marrow cell suspension: (**A**) *the scheme of producing of hemopoietic colonies in the spleen (A), in the mesentery (B), on the CAM (C).* (**B**) *Total mesentery preparation.* (**C**) *Fragment of the colony. (Magnification 4000x).*

Prior to these experiments, we used the Dexter's system to simulate the situation characteristic for xenogeneic (rat in mouse) radiation chimeras, when the rat hemopoietic cells proliferate, differentiate, and attain functional maturity in the stroma of mouse hemopoietic organs (Khrushchov *et al.*, 1987). Bone marrow fragments of F_1(CBAxC57Bl) mice were explanted to form an adhesive cell underlayer *in vitro*. Three weeks later, a Wistar rat bone marrow cell suspension ($10x10^6$ cells per ml) was added. Proliferation and differentiation of the rat hemopoietic cells in such cultures continued for more than 12 weeks (the maximum period of observations). As in conventional long-term bone marrow cell cultures, differentiating cells belonged to the leukocyte and macrophage lineages (Figs. 10A,B,C). The Till and McCulloch's method of spleen colonies allowed us to demonstrate the presence of stem cells (CFU-s) in the cultures throughout the period of observations. Karyotyping, testing for alkaline phosphatase in granulocytes, and reaction with species-specific antibodies against the rat bone marrow cells confirmed the rat origin of these hemopoietic cells.

Subsequent experiments were aimed to reproduce the model of multicomponent xenogeneic chimeras in Dexter's cultures. The mixtures of mouse and rat (1), rat and golden hamster (2), or mouse, rat, and golden hamster (3) bone marrow cells ($1x10^6$ cell of each species per ml) were plated on an adhesive underlayer formed by the mouse bone marrow cells (Fig. 10D). Hemopoietic cells of different genotypes coexisted in such cultures for more than four months (the maximum period of observation), none of them being eliminated. We observed no more than periodic changes in

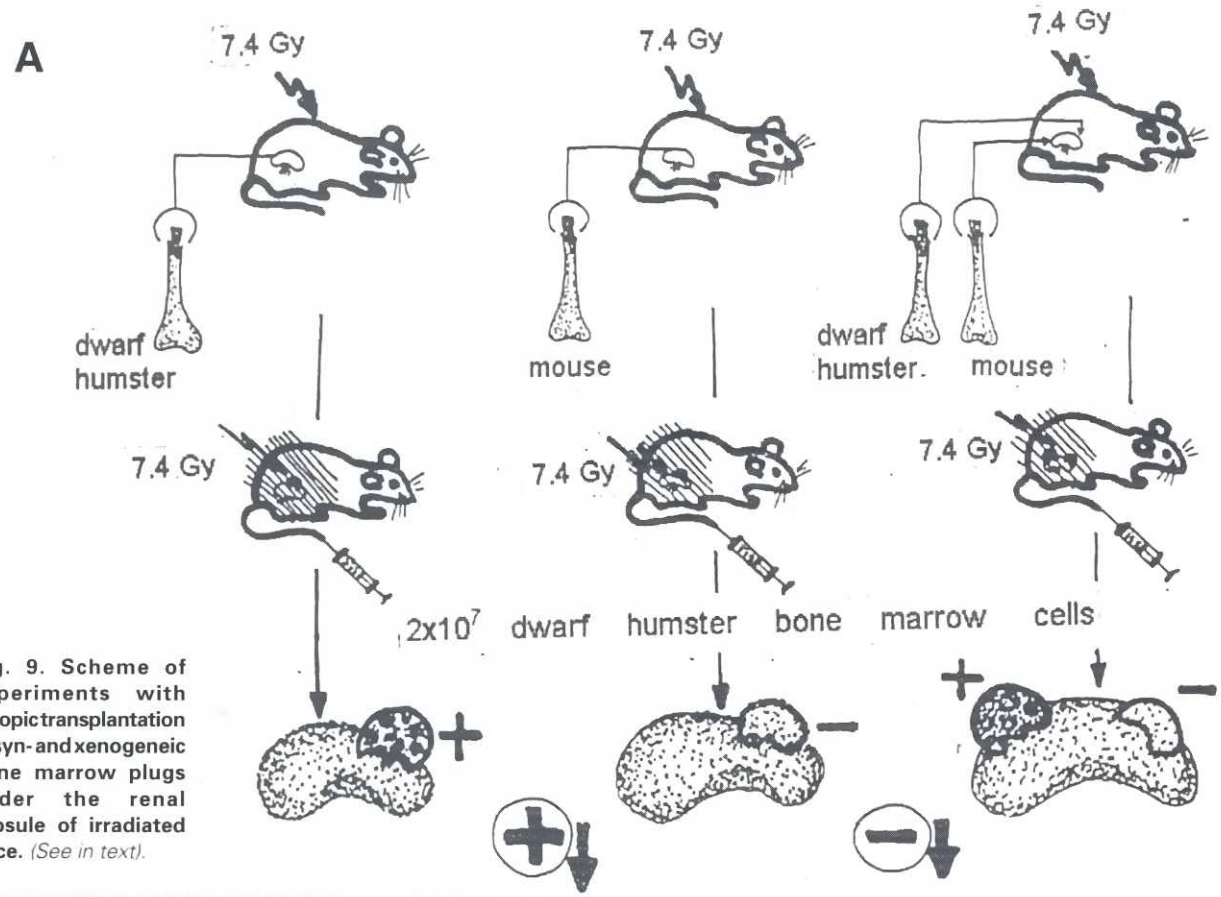
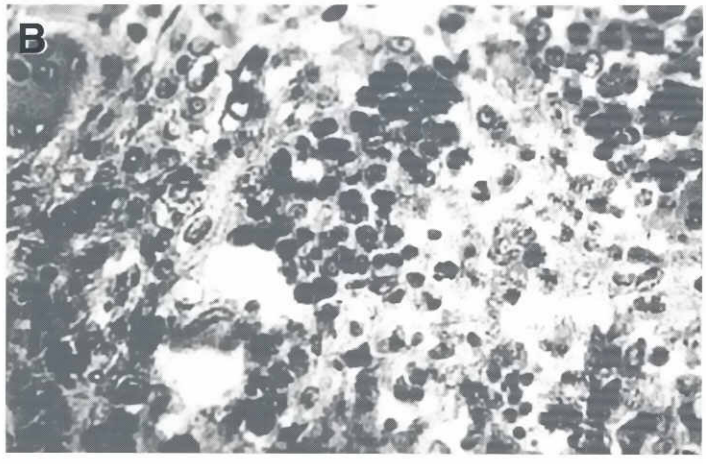
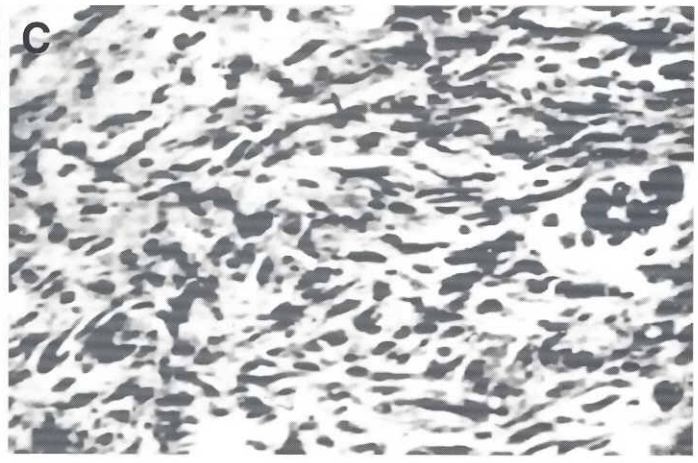


Fig. 9. Scheme of experiments with ectopic transplantation of syn- and xenogeneic bone marrow plugs under the renal capsule of irradiated mice. *(See in text).*





the ratio of hemopoietic cells from different species, which sometimes resulted in the predominance of cells xenogeneic for the stroma.

Therefore, competition between hemopoietic cells of different genotypes observed *in vivo* (in irradiated recipient mice) is missing under the conditions of Dexter's cultures. A possible explanation for this fact is that the culture medium contains excessive amounts of growth factors from horse serum, which are equally foreign to hemopoietic cells of any rodent species. It is apparent, however, that both long-term coexistence of genetically different hemopoietic cells and their rapid competitive displacement will find a more adequate explanation in subsequent studies on the mechanisms of interaction between the hemopoietic cells and stroma.

Myeloleukemia HL-60 and erythroleukemia K-532 cell transplantation to irradiated mice with implanted CAMs

We analyzed the capacity of erythroid or myeloid leukemia cells (K-532 or HL-60, respectively) to proliferate and differentiate on a stromal cell underlayer formed on CAMs in the peritoneal cavity and mesentery. In planning these experiments, the data obtained by Ohkava and Harigaya (1987), and Kodama *et al.* (1991) were taken into account. They suggest that myeloid leukemia cells can attach to the bone marrow stromal cells; on the

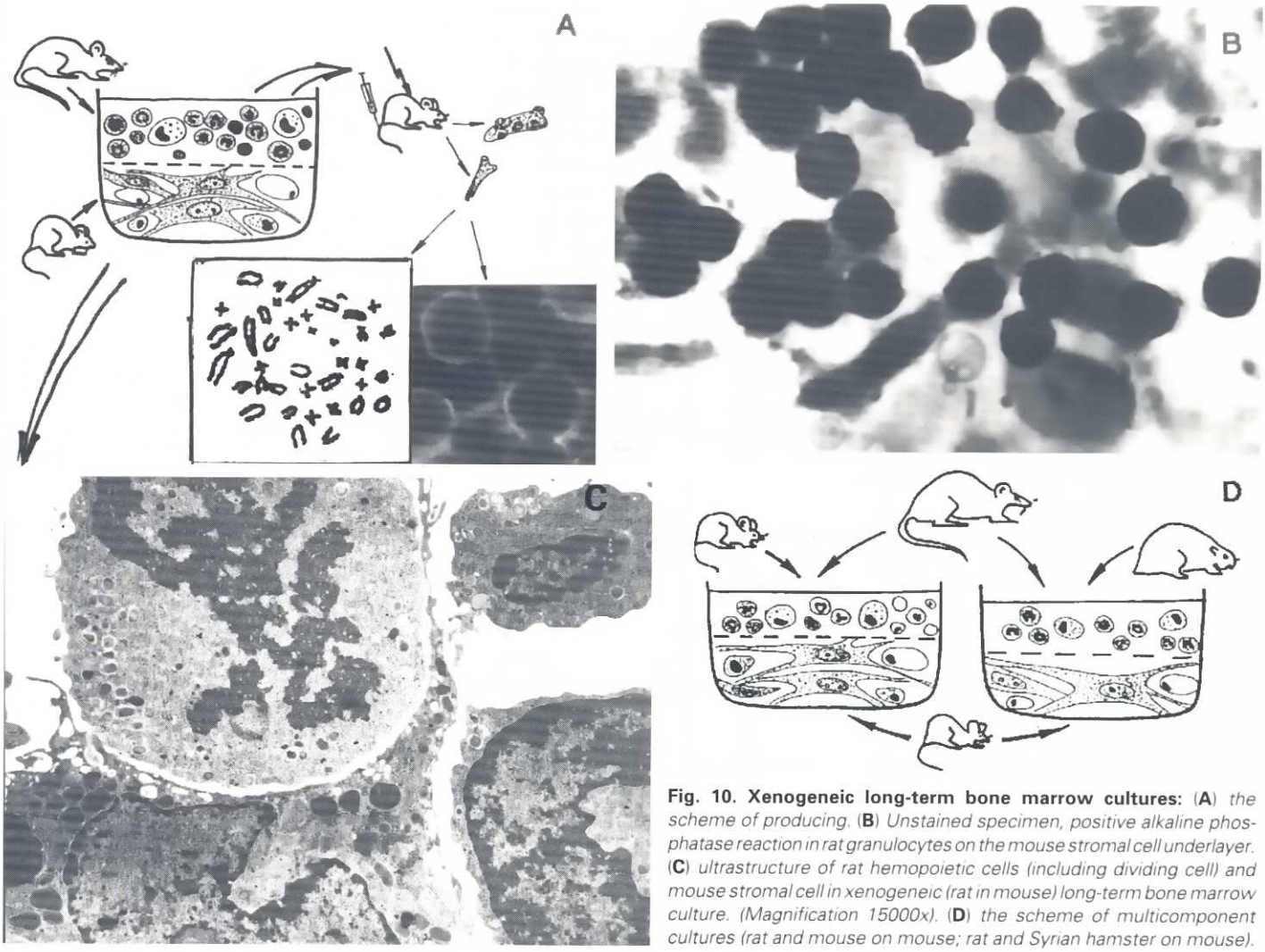


Fig. 10. Xenogeneic long-term bone marrow cultures: (**A**) *the scheme of producing.* (**B**) *Unstained specimen, positive alkaline phosphatase reaction in rat granulocytes on the mouse stromal cell underlayer.* (**C**) *ultrastructure of rat hemopoietic cells (including dividing cell) and mouse stromal cell in xenogeneic (rat in mouse) long-term bone marrow culture. (Magnification 15000x).* (**D**) *the scheme of multicomponent cultures (rat and mouse on mouse; rat and Syrian hamster on mouse).*

other hand, no attachment was observed between HL-60 cells and stromal cells from the human thymus or lymph node, or between lymphocyte leukemia cells and bone marrow stromal cells.

One week after CAM implantation into the peritoneal cavity, the mice were irradiated at 6.5 Gy and received intraperitoneal injections of $1x10^5$ to $1x10^6$ HL-60 or K-532 cells. Six to eight days later, a small number of colonies, each consisting of 50-100 poorly differentiated blast-like cells, formed on CAMs and in the mesentery of recipients. No such colonies developed in the control (noninjected) mice.

Hemopoiesis in experiments with underlayers of different cell composition

In the experiments described above, we used CAMs implantation into the peritoneal cavity of irradiated recipient mice to study hemopoiesis on the fibroblast (fibroblast-like) cell underlayers formed as a result of inflammation. The next experimental series dealt with analyzing hemopoiesis on stromal underlayers of different cell composition, formed under different conditions by stromal cells of different origin. To perform this study, we developed an original experimental model. The first step was to form different cell underlayers on CAMs *in vitro*. These CAMs were then implanted into the peritoneal cavity of recipient mice. The latter were irradiated three to five days before implantation to prevent the host fibroblast precursors from contributing to stromal cell underlayers. In half of the cases, the membranes with underlayers were also irradiated before implantation. Two to three hours after CAMs were implanted, the recipients received intraperitoneal injections of nucleate bone marrow cells from syngeneic donors ($1-5x10^6$ cells each), (Fig. 11). After five to seven days, CAMs were removed, tested for myeloperoxidase and hemoglobin (benzidine reaction), fixed, and stained to histologically analyze hemopoietic foci formed on different cell underlayers. Some CAMs were examined under an electron microscope.

We tested stromal cell underlayers formed by the following cell types: (1) mouse embryonic and fetal fibroblasts (days 7, 12, 13, 15, and 17 of development); (2) skin, liver, and bone marrow fibroblasts of 17-day mouse fetuses and newborn mice; (3)

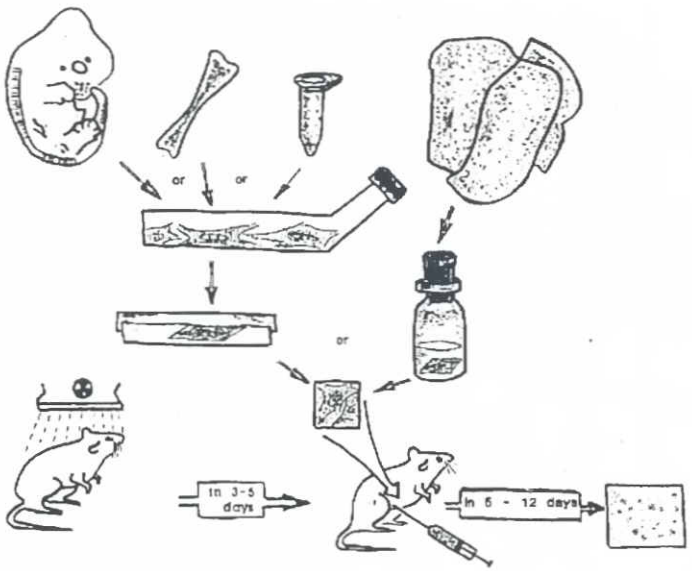


Fig. 11. The scheme of experiments with underlayers of different cell composition.

fibroblasts from the monolayer cultures of mouse and rat bone marrow; (4) fibroblasts of several lines; (5) hepatocytes of 17-day mouse fetuses; (6) hepatocytes of sexually mature rats; and (7) cells transgenic for growth factor genes.

Hemopoietic foci on embryonic fibroblast underlayers

Mouse embryos and fetuses were obtained by mating CBA females to C57Bl males, stages of development being determined from the day of mating. After removing the head and liver, embryonic tissues were minced, treated with trypsin, and filtered through a nylon gauze. The resulting cell suspension was washed many times and introduced into plastic cell culture flasks containing Eagle's medium supplemented with 10% fetal calf serum (0.5-1x10^6 cells per ml). Embryonic fibroblasts were subcultured in the same medium every three to five days, at a concentration of 1-2x10^5 cells per ml. After the third or fourth passage, embryonic fibroblast suspension (2x10^5 cells per ml) was transferred into the flasks containing pieces of Millipore filter (pore size 0.45 μm, one 10x20 mm piece per flask) and cultivated there for three to ten days, the medium being changed every three days. Filters with fibroblast cover were implanted into the peritoneal cavity of F_1(CBAxC57Bl) hybrid mice irradiated at 6.5 Gy three to five days prior to this operation. Syngeneic donor hemopoietic cells were injected intraperitoneally two to three hours after implanting Millipore filters.

We detected no hemopoietic foci on underlayers formed by fibroblasts of 7-day mouse embryos and only occasional foci of poorly differentiated hemopoietic cells on underlayers consisting of 12-day mouse embryonic fibroblasts.

Fibroblasts of 13- and 15-day embryos formed underlayers supporting the development of erythrocyte, granulocyte, mixed colonies and colonies of poorly differentiated blast-like cells. We also detected single mast cell colonies. Embryonic fibroblasts of different passages showed no significant differences with respect to their ability to support hemopoiesis or to the lineage of differentiating hemopoietic cells.

Hemopoietic foci on underlayers formed by fibroblasts from the skin, liver, and limbs (with bone marrow) of 17-day embryos or newborn mice

Cell suspensions were prepared from the skin, limbs, and liver of embryos and newborn mice using the above described method. Liver cell suspension was added to plastic cell culture flasks to a concentration of 2x10^6 cells per ml. The medium was changed on the next day, and cells were first transferred to new flasks on day 10 (0.7x10^5 cells per flask) and cultivated in the medium replaced every three days. After 10-day cultivation on fibroblasts of the third passage (0.2x10^5 cells) were plated on filters. Ten days later, the filters covered by fibroblasts from the liver cell suspension were implanted into the intraperitoneal cavity of irradiated recipient mice.

Skin fibroblasts of 17-day embryos supported growth of hemopoietic colonies of the granulocyte, erythroid, and mixed lineages. In addition, poorly differentiated cell colonies and occasional mast cell colonies developed. On underlayers formed by the fibroblasts from limbs, we detected mainly granulocyte and granulocyte-macrophage colonies. Liver fibroblast underlayers supported the formation of mainly granulocyte and erythroid colonies.

Hemopoiesis on underlayers formed by fibroblasts from monolayer cultures of mouse and rat bone marrow

To obtain monolayer cultures, bone marrow cell suspension was introduced into plastic culture flasks containing Fisher's (Flow Laboratories or Gibco) or alpha-MEM (Sigma) medium with 10% fetal calf serum (1x10^6 cells per ml). The medium was changed after seven days. The fibroblast colonies formed on day 11-14 were removed by trypsinization. The resulting cell suspension was transferred into plastic vessels (1x10^5 cells per ml). Fibroblasts of the second to eighth passage were plated on filters to form a monolayer. The filters were then implanted into the peritoneal cavity of irradiated mice. In one experiment, monolayer cultures of Wistar rat bone marrow fibroblasts were obtained. Filters with rat fibroblast monolayers were likewise implanted into mice, but in this case the recipients were intraperitoneally injected with the Wistar rat hemopoietic cells.

Hemopoietic colonies developing on fibroblast underlayers derived from monolayer cultures of the mouse and rat bone marrow were mainly of the granulocyte lineage. In addition, we detected erythroid cell foci, colonies of poorly differentiated cells, and mixed colonies.

3T3 Fibroblasts used as a stromal underlayer

On underlayers formed by 3T3-Swiss fibroblast line, we occasionally observed small foci of no more than 50-100 poorly differentiated cells (donor hemopoietic cells were obtained from F_1(CBAxC57Bl) mice). According to Roberts *et al.* (1987), however, 3T3 cells can support *in vitro* proliferation and differentiation of pluripotent spleen colony-forming cells obtained by fluorescence-activated cell sorting of IL-3-dependent pluripotent stem cell lines. In another experimental series, we fixed filters with fibroblast underlayers in 1% glutaraldehyde and thoroughly washed them prior to implantation into the peritoneal cavity of recipient mice. In these experiments, different types of hemopoietic colonies developed: granulocyte, erythroid, poorly differentiated, and mixed. Other authors also observed that glutaraldehyde-fixed

underlayers formed by certain cell lines can maintain hemopoiesis *in vitro* (Roberts *et al.*, 1987; Yanai *et al.*, 1989; Kodama *et al.*, 1991). This suggests evidence that molecules associated with the stromal cell membrane or extracellular matrix, rather than soluble cytokines produced by stromal cells, play the key role in producing hemopoietic microenvironment.

Development of different types of hemopoietic colonies on fixed 3T3 cells in the absence of hemopoiesis on live 3T3 cell underlayer requires further discussion. The latter result may be associated with the fact that live stromal cells were not syngeneic to donor hemopoietic cells. On the other hand, it is probable that fixed 3T3 cells provide no more than supporting substructure for hemopoietic cells, whereas growth factors necessary for hemopoiesis come from the peritoneal exudate of irradiated recipients.

If the filters covered by live 3T3 cells were treated with lipopolysaccharide (LPS) from *E. coli* (2 ng/ml) one day before their implantation into the peritoneal cavity, on such underlayers numerous granulocyte colonies and several erythroid and poorly differentiated hemopoietic foci were formed. This result should be interpreted with an account of the data obtained by Rennick *et al.* (1987). In their experiments, Gy30 cells stimulated with 2 ng/ml LPS produced approximately 40 U/ml of GM-CSF, whereas untreated cells produced less than 1 U/ml. LPS is also known to stimulate the marrow stromal cell and mouse stromal cell lines to produce activin A, which induces erythroid differentiation (Shao *et al.*, 1991,1992; Yu *et al.*, 1991,1994; Yamashita *et al.*,1992).

Peculiarities of hemopoiesis on hepatocyte-containing stromal cell underlayers

Pieces of Millipore filters were placed into culture flasks filled with the liver cell suspension obtained from 17-day embryos or newborn mice ($15x10^6$ cells per ml). The medium was changed on the next day. Filters cultivated for two to three days and covered by hepatocytes were implanted into the peritoneal cavity of irradiated mice, which then received intraperitoneal injections of $1\text{-}5x10^6$ syngeneic bone marrow cells. Examination of cell underlayers showed that hepatocytes, which included partially degenerated cells, became covered by one week later one or two fibroblast layers, on which hemopoietic cells were located. The origin of these fibroblasts (donor or recipient) remains unclear. Note, however, that hepatocyte underlayers implanted into some control animals (not injected with hemopoietic cells) also became covered by the fibroblasts originating from radioresistant recipient precursors.

Such hepatocyte-containing underlayers supported formation of large clusters of poorly differentiated cells with mitoses (Fig. 12). No differentiated hemopoietic cells were found. We detected only few groups of poorly differentiated granulocytes (myelocytes and metamyelocytes) and many mast cells, mostly juvenile. Neither blast-like nor mast cells were detected on the filters implanted into control animals (not injected with the bone marrow cells).

Similar results were obtained when analyzing hemopoiesis on the underlayers containing mature rat hepatocytes, after injection of the syngeneic rat bone marrow cells. Hepatocytes were isolated from the liver of mature male Wistar rats weighing 120-160g, after its two-stage perfusion with (1) calcium-free Hanks solution containing 0.5 mM EGTA and 10 mM HEPES and (2) medium 199 containing 0.05% collagenase and 10 mM HEPES. After removal of the liver capsule, the tissue was homogenized and repeatedly

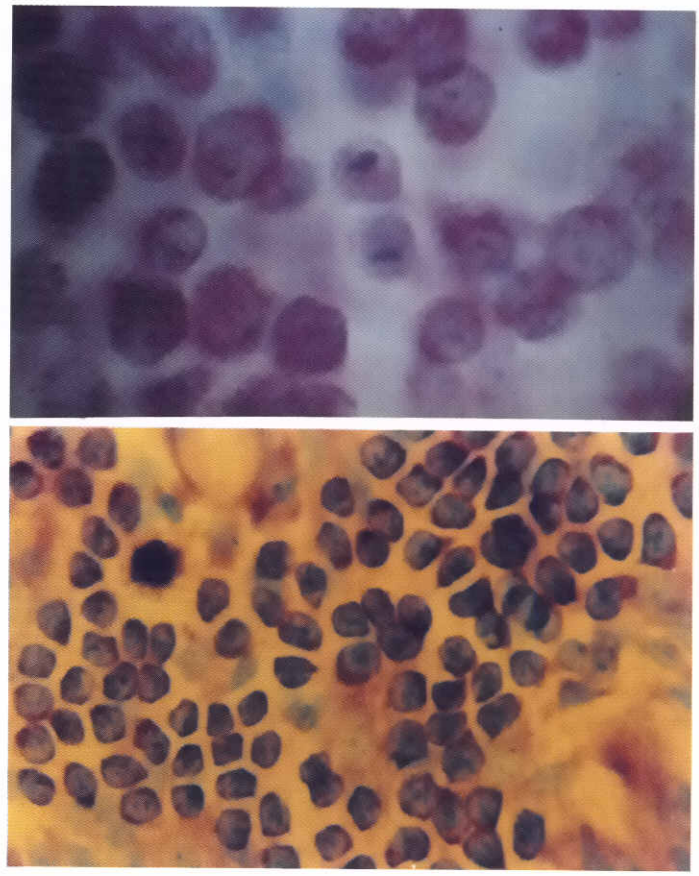


Fig. 12. Blast colonies (foci) formed on hepatocyte-containing underlayer.

filtered through the nylon gauze with subsequent centrifugation at 0°C to prepare suspension of parenchymal liver cells. The resulting preparation consisted of hepatocytes (95% undamaged) suspended in medium 199 supplemented with 20% fetal calf serum [$(10(20)x10^6$ cells per ml]. Pieces of Millipore filters were incubated in this suspension for 4 h. After changing the medium, the filters were cultivated for two more days for the hepatocyte monolayer to develop. The filters covered by hepatocytes were implanted into the peritoneal cavity of irradiated mice, which then received intraperitoneal injections of $(10\text{-}15)x10^6$ rat bone marrow cells (syngeneic to the underlayer hepatocytes). Numerous foci of intensely proliferating poorly differentiated cells were detected on such underlayers.

Note that the stem cell factor (SCF; also referred to as Mast Cell Growth Factor or Steel Factor) was first isolated from the medium conditioned by rat hepatocytes (Zsebo *et al.*, 1990). The presence of this factor in hepatocyte-containing underlayers may account for the formation of mast cell foci as well as for certain peculiarities of hemopoiesis observed in our experiments.

Hemopoietic foci on underlayers formed by newborn mouse kidney cells

CAMs were incubated in kidney cell suspension ($3x10^5$ cells per ml) obtained from newborn F_1(CBAxC57Bl) mice for 24 h. After replacing the medium, incubation continued for one to seven days to obtain primary monolayer cultures of the kidney cells on

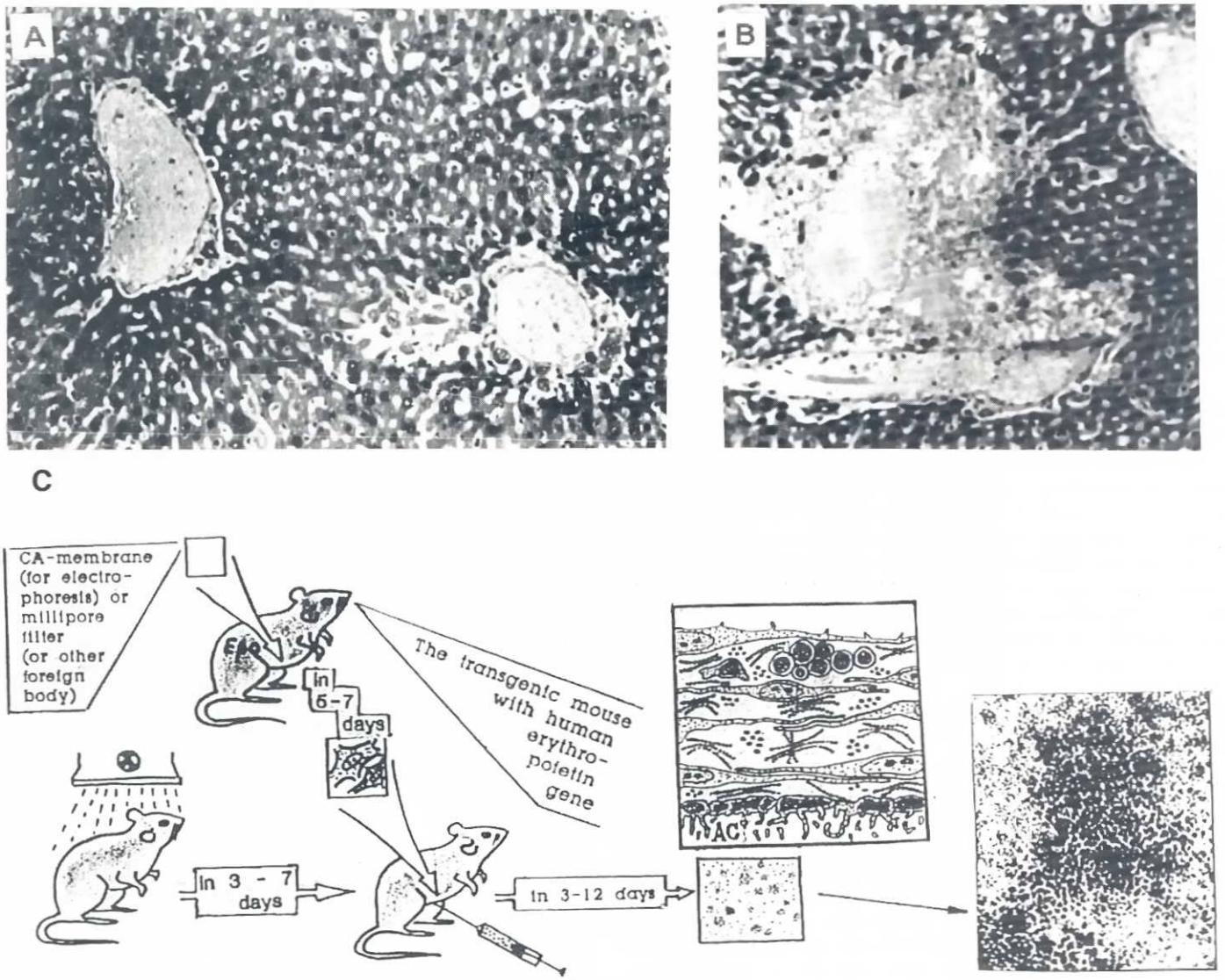


Fig. 13. Experiments with mice transgenic for Epo gene: (**A**) *blood vessels filled with densely packed red blood cells in the liver of the transgenic mouse.* (**B**) *Some pathological changes around congested blood vessels in the liver of transgenic mouse.* (**C**) *The scheme of experiments with transgenic cell underlayers on CAM's.*

CAMs, and the latter were then implanted into recipient mice. Erythroid colonies and clusters prevailed on such underlayers. In addition, we detected granulocyte foci of different size and small colonies of poorly differentiated cells. Prevalence of erythroid colonies may be due to erythropoietin (Epo) synthesis by the epithelial kidney cells, although there is evidence that the erythropoietin gene (*Epo*) in newborn mice is expressed mainly in the hepatocytes (Eckardt *et al.*, 1994).

Hemopoiesis on stromal cell underlayers transgenic for the erythropoietin gene

Transgenic mice were produced using a recombinant gene construct consisting of 5'-promoter-enhancer region of mouse metallothioneine I gene, the coding part of human chromosomal Epo gene (*EPO*), and 3'-region of cattle growth hormone gene containing signals for correct splicing and polyadenylation. A linear DNA fragment, free from any plasmid sequences, was microinjected into mouse embryos, from which 28 mice developed. The Epo activity in blood serum was estimated using a system of blast cells from the mouse spleen. High Epo levels were detected in three mice and in eight their descendants. Analysis of DNA (from the tail) showed that some of these animals carried copies of the injected recombinant construct integrated in their genomes (Kuzin *et al.*, 1991; Khrushchov *et al.*, 1994).

Histological analysis of different organs revealed numerous disorders in transgenic mice, including polycytemia; dilated blood vessels filled with densely packed red blood cells, particularly in the liver, lungs, and kidneys; some pathological (necrotic) changes around the congested blood vessels; foci of extramedullary hemopoiesis in the liver, leg skeletal muscle, and around certain blood vessels; numerous mast cells in lymph nodes; etc. (Fig. 13A,B).These observations agree with the published data on histological changes in transgenic mice expressing the human *EPO* gene (Semenza *et al.*, 1989; Metcalf, 1990).

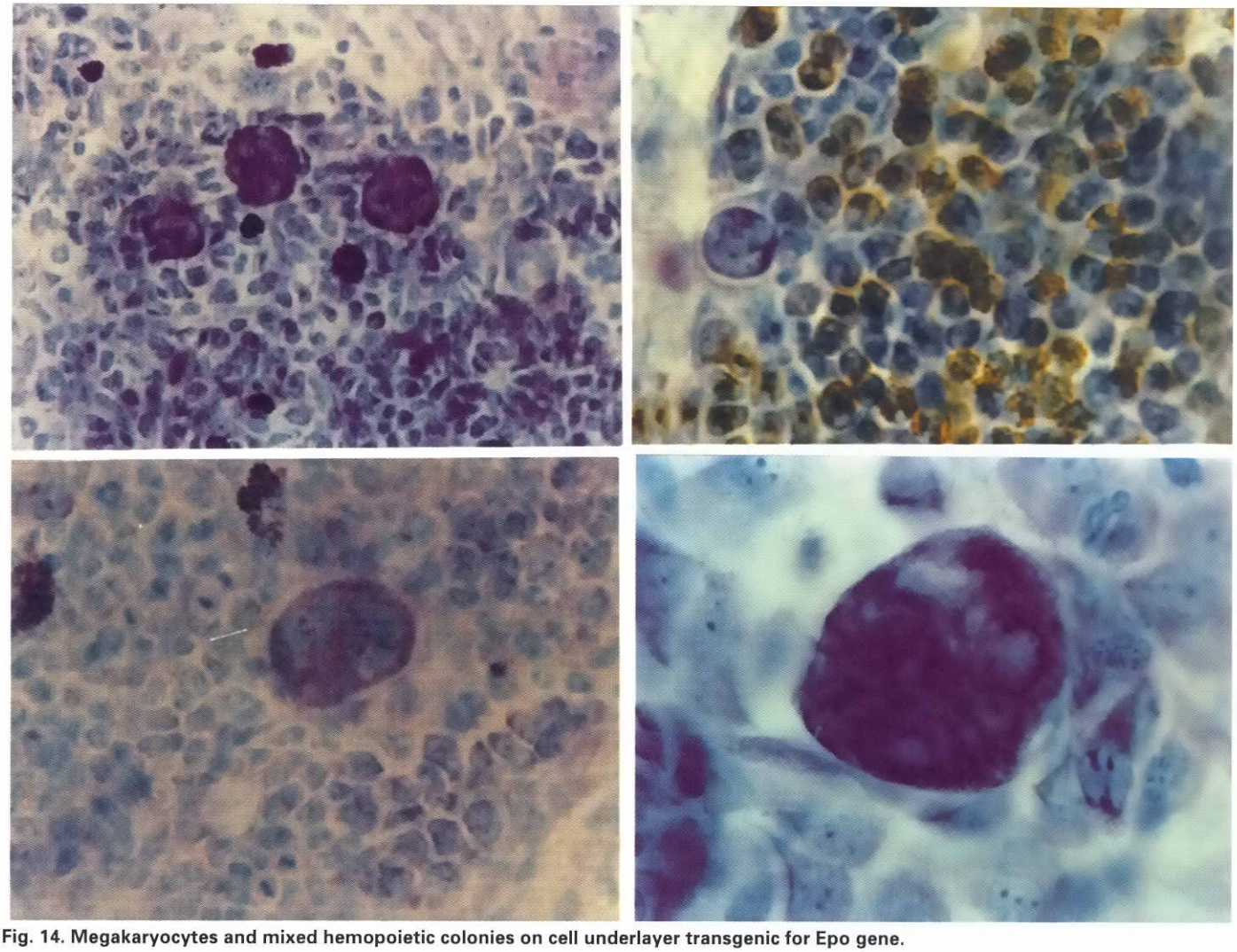


Fig. 14. Megakaryocytes and mixed hemopoietic colonies on cell underlayer transgenic for Epo gene.

To study the effect of transgenic stromal cells on differentiation of hemopoietic clonogenic cells, we implanted CAMs into the peritoneal cavity of transgenic mice. Seven days later, after the formation of transgenic cell underlayer, CAMs were transferred into the peritoneal cavity of nontransgenic mice irradiated at doses of 6.1-7.0 Gy three to five days before this operation to prevent the involvement of their fibroblast-like cells in forming the underlayer. After transferring CAMs, nontransgenic recipients were injected with donor hemopoietic cells, and transgenic mice received fresh implants. Having repeated implantation of fresh CAMs five to seven times, we managed to obtain ample experimental material (transgenic underlayers on CAMs) from few transgenic mice (Fig. 13C). The latter were then sacrificed, and their bone marrow was transferred *in vitro* to establish long-term bone marrow cell culture (LTBMC) producing adhesive cell underlayers.

LTBMCs provide a convenient *in vitro* model of myeloid differentiation. Hemopoietic stem cell self-renewal and differentiation into granulocytes occur in association with the inductive microenvironment consisting of multiple cell types. Mature granulocytes are continuously produced for several months, as long as an appropriate balance between the pluripotent stem cells, CFU-gm and more differentiated myeloid precursors, and mature cells is maintained. Erythropoiesis does not generally proceed past the most primitive BFU-e. Differentiated progeny (CFU-e) and morphologically recognizable erythroid cells do not appear unless LTBMC is supplemented with exogenous Epo, and sustained erythroid differentiation is impossible without repeatedly adding this factor to the culture medium.

Three weeks after explanting transgenic bone marrow *in vitro*, we plated the bone marrow cell suspension from normal mice to transgenic stromal cell underlayer, according to Dexter. In these cultures, we observed macroscopically evident erythroid differentiation with clumps of hemoglobin-containing red blood cells. Hemoglobinization was confirmed by benzidine staining of nonadherent cells, which showed that up to 40% of nucleate cells were benzidine-positive. Epo was also present in the culture medium.

As noted above, stromal cell underlayers formed on CAMs in the peritoneal cavity of mice support differentiation of transplanted bone marrow cells mainly into granulocytes. However, when CAMs were implanted into mice transgenic for *EPO*, we

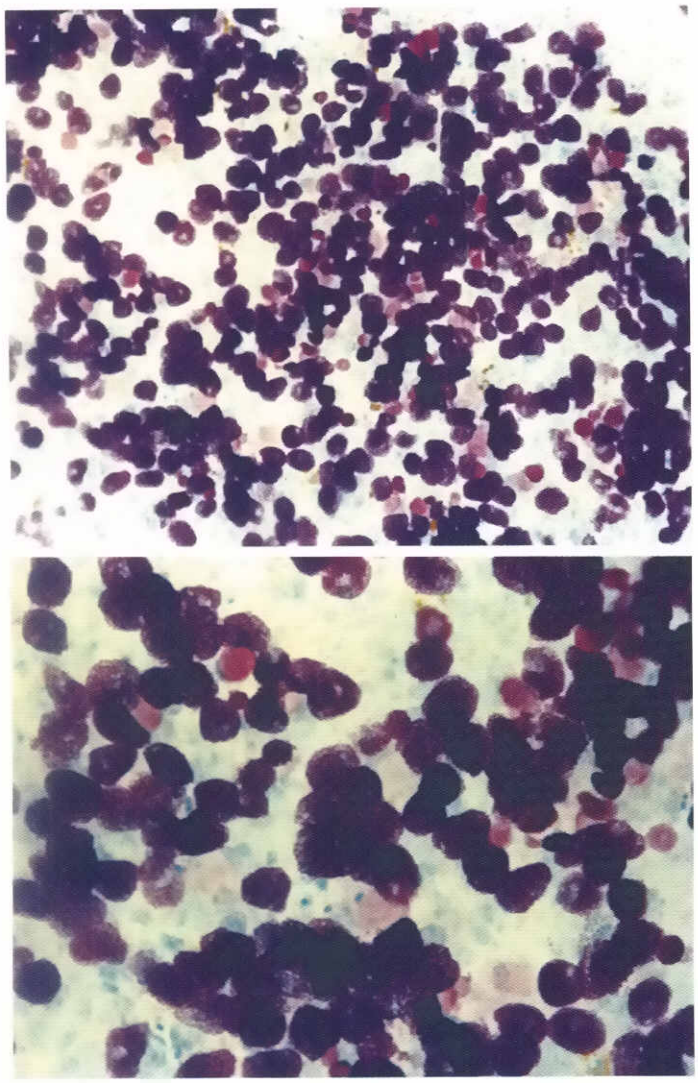


Fig. 15. The mast cell colony on cell underlayer transgenic for Epo gene.

detected numerous erythroid colonies on these underlayers, which often contained megakaryocytes (Fig. 14). Some colonies consisted of megakaryocytes only. Others were formed by poorly differentiated cells, granulocytes, and mast cells. There were many colonies of the latter type, and some of them included more than 1000 cells (Fig. 15).

Similar features of hemopoiesis were observed on CAMs implanted into the peritoneal cavity of normal (nontransgenic) mice with Epo levels artificially increased by bleeding or injections of exogenous Epo. In experiments with bleeding, we used normal F_1(CBAxC57Bl) or CBA mice. Six days after implanting CAMs, 0.2-0.5 ml of blood (10-25% of the total blood volume) were removed from each recipient through the tail vein. Mice were irradiated and injected with the donor bone marrow cells 18-24 h after bleeding, when the serum Epo concentration attained its peak. Colonies on CAMs were analyzed seven days later. In another experimental series, mice with implanted CAMs received injections of exogenous Epo (Erythropoietin Human Recombinant, Sigma, or Erythropoietin Lyophilisate, Boehringer Mannheim GmbH) 1 or 2 h before bone marrow cell transplantation. Epo doses of 2.5, 5, or 25 I.U. were used.

Erythroid differentiation of hemopoietic cells in these experiments, as well as on the *EPO*-transgenic stromal underlayers formed in LTBMC and on CAMs in the peritoneal cavity, is readily explainable. This result agrees with the data of Corey *et al.* (1990), who observed up to 70% of erythroid (benzidine-positive) cells in LTBMC after introducing human Epo cDNA into the hemopoietic microenvironment. Differentiation of transplanted hemopoietic cells into megakaryocytes under the conditions of excessive Epo concentration appears also natural, because the stimulatory effect of Epo on megakaryocyte differentiation was repeatedly demonstrated. Thus, recombinant human Epo (rHuEpo) stimulated clonal growth of the human and mouse megakaryocyte colonies or isolated megakaryocytes *in vitro* (Dukes *et al.*, 1986; Dessypris *et al.*, 1987; Ishibashi *et al.*, 1987) and exerted a direct effect on the rat bone marrow megakaryocytes and their immediate precursors (Berridge *et al.*, 1988). Fraser and his colleagues reported specific binding of ^{125}I-labeled recombinant Epo to human megakaryocytes (Fraser *et al.*, 1988; Hoffman, 1989) and expression of specific high-affinity Epo binding sites on the rat and mouse megakaryocytes (Fraser *et al.*, 1989; Jones *et al.*, 1990). Moreover, it has been shown that rHuEpo stimulates the formation of mixed erythroid-megakaryocyte and blast cell colonies, as well as erythroid colonies, erythroid bursts, and megakaryocyte colonies, from the normal mouse bone marrow cells, i.e., functions not only as a late-acting factor specific for erythroid progenitors but also as a bipotential erythroid-megakaryocyte-stimulating factor for the mouse hemopoietic cells (Nishi *et al.*, 1990). We cannot rule out the possibility that Epo has a similar effect on the mast cell precursors and is involved in proliferation and differentiation of this cell type. It was shown that endothelial cells have erythropoietin receptors and respond to Epo with enhanced proliferation (Anagnostou *et al.*, 1990; 1994). The concept of strict erythroid or even hematopoietic specificity of EpoR is being challenged not only by these studies but also those of Masuda *et al.*, (1993), who described the presence of EpoR in two rodent cell lines of neural origin. According to Anagnostou *et al.* (1994), Ohneda *et al.* (1993) observed a dose-dependent mitogenic response of murine fetal liver stromal cells to Epo and detected EpoR mRNA in those cells. Heberlein *et al.* (1992) demonstrated low levels of EpoR gene transcripts in embryonal or multipotential cell lines. These primitive cells had 10-100 times less EpoR mRNA than mature erythroid precursors. In fact, the EpoR gene seems to be expressed at low levels before either hematopoietic or erythroid commitment has occured (Schmitt *et al.*, 1991; Heberlein *et al.*, 1992), giving rise to speculation that EpoR in these early cells can transmit a proliferative signal either by itself or in synergy with other cytokine receptors (Heberlein *et al.*, 1992; Anagnostou *et al.*, 1994)

Should subsequent experimental research disprove the presence of Epo receptors on mast cells, we could offer another, as yet speculative explanation: increased Epo concentrations and the development of erythroid and megakaryocyte colonies correlate with a local increase in concentrations of the other growth factors (IL-3, SCF, etc.), and these combined effects promote the formation of mast cell colonies.

In conclusion, we must admit that our numerous experiments have not yet revealed fine molecular mechanisms of cell interactions during hemopoiesis. Nevertheless, we hope that our experimental models and the data obtained with their aid will help in gaining new knowledge in this field. It is apparent that the choice of an adequate experimental model is very important for properly applying powerful methods of modern molecular biology, molecular genetics, immunology, and other disciplines in solving the current problems of cell and developmental biology.

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