Liver regeneration: effect of spaced resections

	Interval after single resection at which cells are removed for culture			
	8 h	24 h	36 h	48 h
a) Single 68% resection	NG	NG	NG	546[a] ± 24
b) Single 30% resection	NG	NG	NG	168 ± 32
	Interval after second resection at which cells are removed for culture			
	8 h	24 h	36 h	48 h
c) 38% resection followed in 24 h by 30+% resection	363[a] ± 37	243 ± 23	396 ± 30	413 ± 33
d) 38% resection followed in 40 h by 30+% resection	67 ± 14	153 ± 27	212 ± 32	214 ± 40
e) 38% resection followed in 72 h by 30+% resection	101 ± 5	140 ± 6	210 ± 6	755 ± 21

[a] Number of cells/plate × 10^3 (mean ± S.D.). NG = no growth. – Cell pools were formed from trypsin-dispersed regenerating liver tissue removed from 5 rats. The inoculum in each plate was 1.8 × 10^6 of these cells. The figure for each time interval (above) represents the mean ± S. D. in 20 plates with the same inoculum cultured for 16 days (see text.).

of cells removed at all 3 intervals, 8, 24, and 36 h following the second resection. As in previous studies[1,2], tissue removed 48 h following any size of resection (or resections) grew in vitro and maximum growth was always obtained in tissue removed at this interval post resection (table). Following the 40 h and 72 h (2nd) resections, cell survival was increased with each lengthening of interval post resection, i.e., 8 h post resection to 48 h post resection.

The relative failure of cells from liver to form a monolayer when they are removed during the initial interval (< 46 h) following 68% hepatic resection, employing this culture technique is of interest, as it is during this precise period that all of the principal indices of the in vivo replication of cells in the remnant liver during the regenerative response reach a peak and decline[3–7]. Cells from regenerating liver tissue cultured in soft agar also have minimal colony forming capacity when they are removed during this initial period following hepatic resection, and actively form colonies when they are removed later in the regenerative response[8,9]. This 'growth inhibition' interval appears to be longer in the mouse than in rats[8,9], as is the general regenerative response.

It might be assumed that this relative growth inhibition was related to the effect of the trauma of the liver excision per se on remnant cells, possibly producing an increased susceptibility to the action of trypsin, or in some other way directly inhibiting capacity for in vitro growth. From these studies however, it appears that this is not the case. The *initial* hepatic resection appeared to be the governing factor in establishing the growth potential of the regenerating liver cells in vitro. A second resection was not followed by a period of growth inhibition, a phenomenon always seen following a single resection.

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Acceleration of muscle regeneration by bone marrow cells[1]

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Summary. Myogenesis was accelerated by addition of bone marrow cells to muscle minces cultured in diffusion chambers. This effect was inhibited by using gold suppressed bone Marrow cells.

It is generally agreed that muscle regeneration after injury is brought about by activated satellite cells and cleaved mononuclei (surrounded by a sarcoplasmic rim), which proliferate, fuse and synthesize myofilaments[2,3]. However, distally arising cells have not been excluded as myoblast precursors, and the present work was undertaken to test such a possibility.

The experiments were done on muscle minces cultured in diffusion chambers either alone or mixed with bone marrow cells. The method is nearer to in vivo conditions and is less technically demanding than conventional tissue culturing[4–7]. The chambers (capacity of 0.13 ml) were made of Millipore filters (0.22 μm and 0.45 μm pore size) cemented to both sides of a lucite ring (13 mm in diameter), with an access hole on the side. 2 chambers were implanted into each peritoneal cavity of young

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mice. The muscle was taken from tongues of albino rats weighing 100–120 g. This source was chosen because of the uniformity of myofiber type and its known rapid in situ regenerative ability[8]. The tongue was cleared of its mucosa and chopped into pieces $^1/_2$ mm^3 or smaller in size, each tongue supplying about 25 chambers with 4–12 muscle fragments in each. Femoral bone marrow taken from the same animal, was mixed with the muscle in half the chambers (about $^1/_2$ million cells suspended in 0.03 ml of TC 199 medium[4]).

Myogenic activity in diffusion chambers

Duration of incubation	No. of chambers with muscle alone	Muscle and bone marrow	Muscle and myocrisin treated bone marrow
Total number of chambers	18	19	9
2 days	3*	3*	1*
4 days	3*	3 (2**)	1*
6 days	7 (2**)	8 (6***)	4*
12 days	3 (2**)	2 (1**)	2 (1**)
18 days	2 (2***)	1 (1***)	1 (1**)

* absent, ** present, *** profuse.

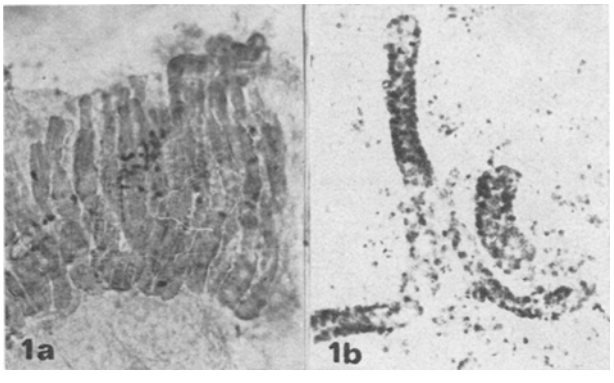

Fig. 1. Muscle fragments as seen on the Millipore filters after 2 days in chamber. *a* Muscle in chamber by itself. × 160. *b* Muscle mixed with bone marrow. The cells aggregate around myofibres. × 180.

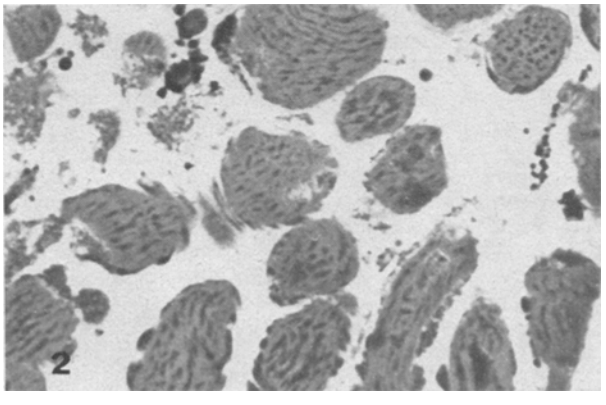

Fig. 2. Fragment of muscle after 6 days alone in chamber. The myofibres look 'inert' with only occasional cells near the fibres. The darker dots are mitochondria. (1 µm section. Epon embedded and toluidine blue stained. × 420.)

In an additional set of experiments, suppression of phagocytic properties of admixed cells was carried out by administering 5 mg of a gold solution (Myocrisin) to a homologous rat, 24 h before taking its bone marrow[7,9]. The chambers were harvested after 2, 4, 6, 12 and 18 days of culture, changing mice every 6 days to avoid filter blockage and antibody influences.

The retrieved chambers contained a variable amount of colourless or straw-coloured gelatinous material, which was processed for light and electron microscopy. The filters too were stained and examined with a light microscope (figure 1a, b). Out of 66 implants 46 were suitable for final analysis (table). Several mice died (they proved very sensitive to cold) and a number of cultures were discarded because of contamination.

Muscle grown alone looked well preserved and rather 'inert' in the first 6 days. Myofibres, blood vessels, nerves and connective tissues were seen surrounded by an extensive network of fibrin and later young collagen (figure 2). Only after 12 or 18 days were myoblasts and myotubes formed, either inside old basement membranes or 'de novo'.

Where the muscle was mixed with bone marrow, most of the 'free' cells, even in the 2-day-cultures, looked like macrophages although variable numbers of erythrocytes,

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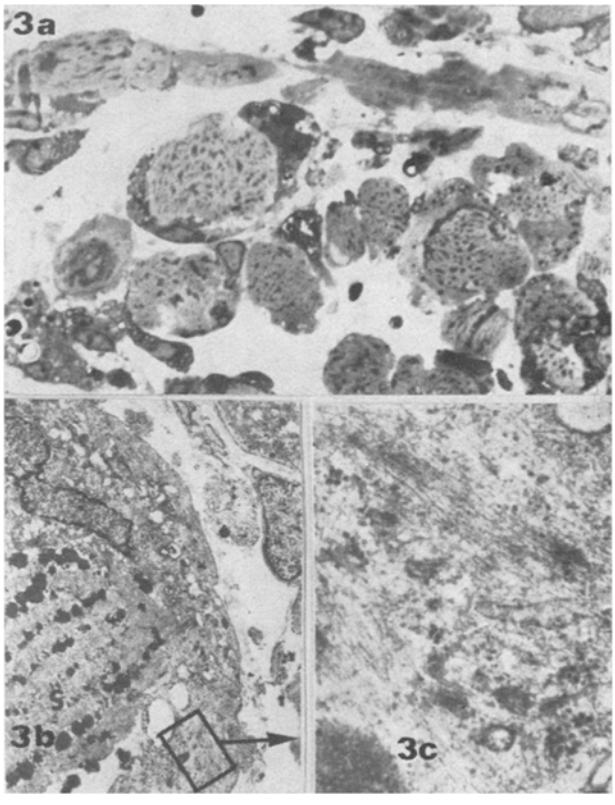

Fig. 3. Muscle with bone marrow cells after 6 days in chamber. *a* There are many interstitial cells and much regenerative activity around the myofibres. × 420. *b* Electron microscopy shows degenerating myofibre with dark mitochondria and sarcoplasmic sap (S). Around it are phagocytic and myogenic cells. × 3000. *c* Enlargement of the rectangular area of *b*, showing thick and thin filaments. × 30,000.

lymphocytes, polymorphonuclear granulocytes (including eosinophils) and fibroblast were also seen. The cells aggregated around muscle fragments (figures 1b and 3a).

Electron microscopy revealed that the apparently well preserved myofibres were in reality in various stages of degeneration. However, where muscle was alone, the degree of disruption was much less marked than at the corresponding period of mixed culture. In the mixed 6-day-cultures, the myofilaments and sarcotubules were matted together into fibrillary or amorphous sarcoplasmic 'sap'. The mitochondria were enlarged, dark, arranged in rows or clumped together. Myonuclei, where recognized, were small, pale with coarse chromatin and a 'blistered' envelope. Within myofibre basement membranes as well as outside them. Frank myoblasts rich in polyribosomes, small pale mitochondria and recognizable thick and thin myofilaments were also seen (figure 3b, c). In many cells, phagocytosis and myofilament synthesis occurred side by side.

In chambers where gold treated bone marrow was mixed with muscle fragments, no myoblastic activity was seen at 6 days of culture, and it was still slight at 12 and 18 days. The gold precipitated in large macrophage-like cells containing multivesicular and heterogeneous bodies[9,10]. These cells were always well separated from the myofibres.

In this study we were not concerned with quantitative factors known to influence proliferation and differentiation[11], but with the effect of distally arising cells on early changes in explanted myofibres. No particular attention was paid to the changes in blood vessels or nerves. Our results indicate that a remarkable acceleration of degenerative and regenerative processes takes place when bone marrow cells are mixed with muscle fragments (table).

The bone marrow derived cells (including polymorphs which soon degenerate and mix with the general pool of debris) are probably responsible for most of the rapid myofibre degradation, although young myoblasts, themselves phagocytic[12], may contribute to a 'mop up' operation. The contractile protein decrease acts as a powerful stimulus to biosynthesis[2] and myoblast formation in the generally accepted ways. After gold treatment, there are not only fewer bone marrow cells but their phagocytosing and metabolic activities are much decreased[7]. Thus, without rapid degeneration no accelerated regeneration occurs.

Another possible, even if far-fetched, interpretation could be that cells originating from bone marrow are themselves induced to transform into myoblasts. This could be brought about by incorporation of material from the degenerating muscle or by fusion with myogenic cells. The paucity of degenerating mononuclear cells, the scarce mitoses and other morphological features suggest that a transition from relatively simple mobile mononuclear cells into large complex forms actively engaged in myogenesis may indeed be taking place. Theoretically, such an interpretation is feasible as macrophages are known to engage in reutilization of ingested products and they apparently can transform into other, even unrelated, forms such as melanoma cells in culture[13].

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Free radicals produced in a nitrosofluorene-unsaturated lipid reaction[1]

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Summary. We report here the first demonstration that the carcinogen 2-nitrosofluorene reacts directly with lipid molecules containing carbon-carbon double bonds to yield free radicals which appear to be the nitroxyl free radical of the carcinogen covalently bound to the lipid.

2-Nitrosofluorene (NOF) is an activated form of the carcinogen 2-acetylaminofluorene (AAF) and produces tumors in mammary gland as well as at the site of subcutaneous injection[2]. NOF can be formed either by deacylation of N-hydroxy-N-acetyl-2-aminofluorene (N-OH-AAF), itself an activated form of AAF, and then subsequent non-enzymatic oxidation[3]; or by the peroxidase or free radical route of N-OH-AAF activation[4–8]. We report here the first demonstration that NOF when exposed to lipid molecules containing a carbon-carbon double bond reacts readily to form a free radical which we postulate is the nitroxyl free radical of the NOF-lipid addition product.

NOF was synthesized as described previously[7]. The synthesized compound was pure by thin layer chromatography and had the same melting point and UV spectrum as reported by Lotlikar et al.[9]. Linoleic acid, oleic acid, octanoic acid and squalene were purchased in their pure forms from Sigma Chemical Co. These were diluted with deoxygenated methanol to a concentration of 2% and stored under nitrogen at −20 °C. Optical surveillance at 233 nm indicated the absence of hydroperoxides. All reactions were carried out in 0.05 M pH 7.4 potassium

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