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The influence of mouse sera, regenerating liver extracts and bacterial products on the abilities of different cells *in vitro*

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ABSTRACT In the complexity of host tumor relations, the regeneration of the tissue in which the tumor is growing, or in some other tissue in the organism, could influence the maturation of tumor cells, *i.e.* tumor reversion. Clinical observations and experiments on plants, lower animals, or animal embryos, performed by several authors, and our results on the influence of regenerating mouse liver on the abilities of tumor transplanted there or elsewhere in the organism led us to study the *in vitro* growth of different cells or bacteria exposed to the extracts of normal or regenerating liver and/or sera from these animals. Further, sterile used bacterial media were added to bacterial or cell cultures, respectively. Depending on the model, liver extracts – particularly extracts and sera from mice with regenerating liver – were shown to inhibit radioactive thymidine incorporation in the cells. In these experiments, the number of bacteria or cells per culture was lower than in otherwise treated corresponding cultures. Further, used sterile media of bacterial cultures stimulated the growth of bacteria but inhibited thymidine incorporation into fibrosarcoma cells *in vitro*. Whether this means that one or several common regulators exist in nature appears as an intriguing, but still completely open question. The idea of controlling tumor growth by using such regulatory growth factors seems very provocative.

KEY WORDS: *regenerating tissue extracts, tumor cell growth in vitro, used bacterial media, partial hepatectomy*

Introduction

Although intensively investigated, the regulatory mechanisms of cell growth and development (cell maturation) are still not entirely understood. This topic is of essential importance, not only for scientists working in different fields of biomedical research, but also for a practical approach to the therapy of various diseases, especially cancer. The possibility of controlling tumor growth by inducing malignant cell differentiation seems very attractive. Numerous factors, such as vitamins, xanthines, growth factors, etc., have been successfully used for this purpose in particular experimental systems (Cho-Chung and Newcomer, 1977; Santoro *et al.*, 1979; Tsuchya, 1982; Bakhanashvile, 1983; Schindler and Sherman, 1984; Pavelic and Spaventi, 1987). However, the usefulness of these agents in cancer therapy is still doubtful (Moore, 1977; Sachs, 1978; Cairns, 1979; Schwartz *et al.*, 1982; Nomura, 1983; Pavelic *et al.*, 1990). On the other hand, the phenomenon of spontaneous cancer regression or even reversion toward normal cells indicates that there are certain unknown mechanisms in the organism which could control tumor growth (Everson and Cole, 1966). Since the spontaneous regression of cancer cannot be generally explained by successful immune reaction of the organism against tumor cells, the mechanism of this phenomenon remains only speculative (Jurin, 1973; Levison, 1976). Tumor regression or the reversion of malignant cells can also be obtained in plants and lower vertebrates by exposing the tumor to the influence of regenerating tissue (Rose and Waligford, 1948; Braun, 1961; Meins, 1977). Furthermore, murine malignant cells can also be induced to differentiate into normal cells if injected into an early stage embryo (McBurney, 1977). Aiming to test whether adult animals also possess the capability of controlling tumor growth, we have analyzed the growth of murine malignant tumors in regenerating liver. During the growth of regenerating liver the anaplasticity of methylcholantrene-induced fibrosarcoma decreased, as well as the incidence of live cells and the intensity of pigmentation in melanoma B16 tissue

Abbreviations used in this paper: CS, calf serum; EGF, epithelial growth factor; FCS, fetal calf serum; HPTA, hepatoprotein A; HPTB, hepatoprotein B; HS, human serum; IL-6, interleukin 6; LE, liver extract; NLE, liver extract from normal mouse; NMS, normal mouse serum; PHLE, liver extract from partially hepatectomized mouse; PHMS, serum from partially hepatectomized mouse; SHLE, liver extract from sham-hepatectomized mouse; SHMS, serum from sham-hepatectomized mouse; Sl saline; TGF ß, transforming growth factor ß.

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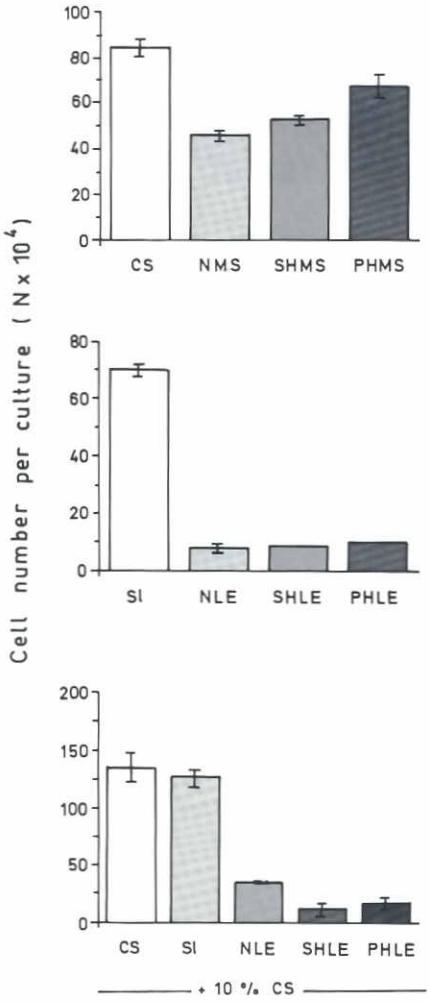

Fig. 1. Numbers of L929 cells following cultivation *in calf serum (CS), normal mouse serum (NMS), sham-hepatectomized mouse serum (SHMS), partially hepatectomized mouse serum (PHMS), normal mouse liver extract (NLE), sham-hepatectomized mouse liver extract (SHLE), partially hepatectomized mouse liver extract (PHLE), combinations of CS and liver extracts, or in medium supplements with an adequate volume of saline (SI), respectively.*

(Zarkovic *et al.*, 1985). Furthermore, it was also noticed that the growth of melanoma B16 transplanted into the hind leg of a syngeneic mouse was inhibited if the host's liver was regenerating after partial hepatectomy (Zarkovic *et al.*, 1990). Similar, but less pronounced tumor growth inhibition was observed in sham-hepatectomized tumor bearing animals. Hence, it has been supposed that tissue regeneration, particularly liver regeneration in mammals, like tissue regeneration in plants or in lower vertebrates, can modify tumor growth features, probably through certain mechanisms *i.e.* factors controlling tissue regeneration. Such factors could be synthesized by regenerating tissue itself, acting as local regulatory factors and systemic factors regulating cell proliferation in remote tissue. To test this hypothesis we have analyzed the effects of liver extracts and/or sera from non-operated, sham-hepatectomized or partially hepatectomized mice on *in vitro* growth abilities of different cell lines, primary cultures of embryonal and tumor cells, and bacterial cultures, respectively.

Results

***The effects of murine sera and liver extracts on cell growth* in vitro**

L929 cells were cultivated *in vitro* in MEM supplemented with calf serum (CS) to obtain a 10% suspension, or an adequate volume of saline. Further, instead of CS, different murine sera, *i.e.* serum from the normal mouse (NMS), sham-hepatectomized (SHMS), or partially hepatectomized (PHMS) mouse were used, respectively. Liver extracts (LE) from normal (NMLE), sham-hepatectomized (SHLE), or partially hepatectomized (PHLE) animals were used alone or in combination with different mouse sera or with CS. The cell numbers in these cultures were determined and the results are presented in Fig. 1. In comparison to the cultures where saline was added, a stimulation of cell growth was observed in CS cultures ($P<0.05$) while suppression was observed in those with NMS ($P<0.05$). Further, SHMS and particularly PHMS stimulated the growth of cells better than NMS ($P<0.05$). On the other hand, all liver extracts used inhibited L929 cell growth and the inhibition was about the same if NMLE, SHLE or PHLE were used, respectively (Fig. 1). Further, LE

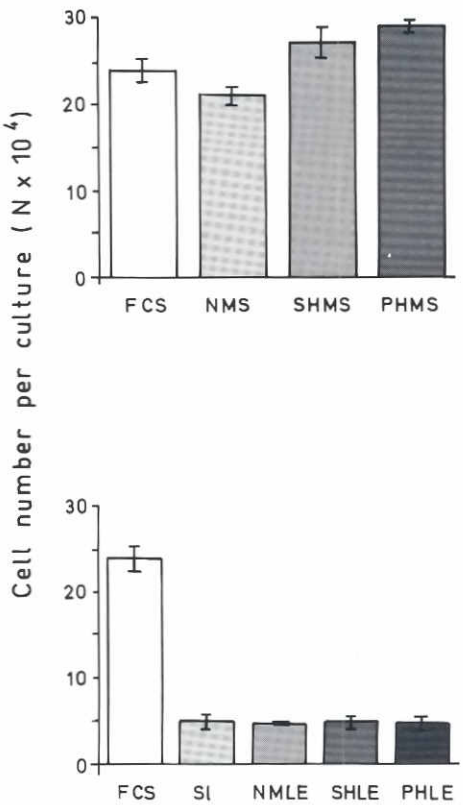

Fig. 2. Numbers of HEp-2 cells following cultivation *in medium with fetal calf serum (FCS), normal mouse serum (NMS), sham-hepatectomized mouse serum (SHMS), partially hepatectomized mouse serum (PHMS), normal mouse liver extract (NLE), sham-hepatectomized mouse liver extract (SHLE), partially hepatectomized mouse liver extract (PHLE) or in medium supplemented with an adequate volume of saline (SI), respectively.*

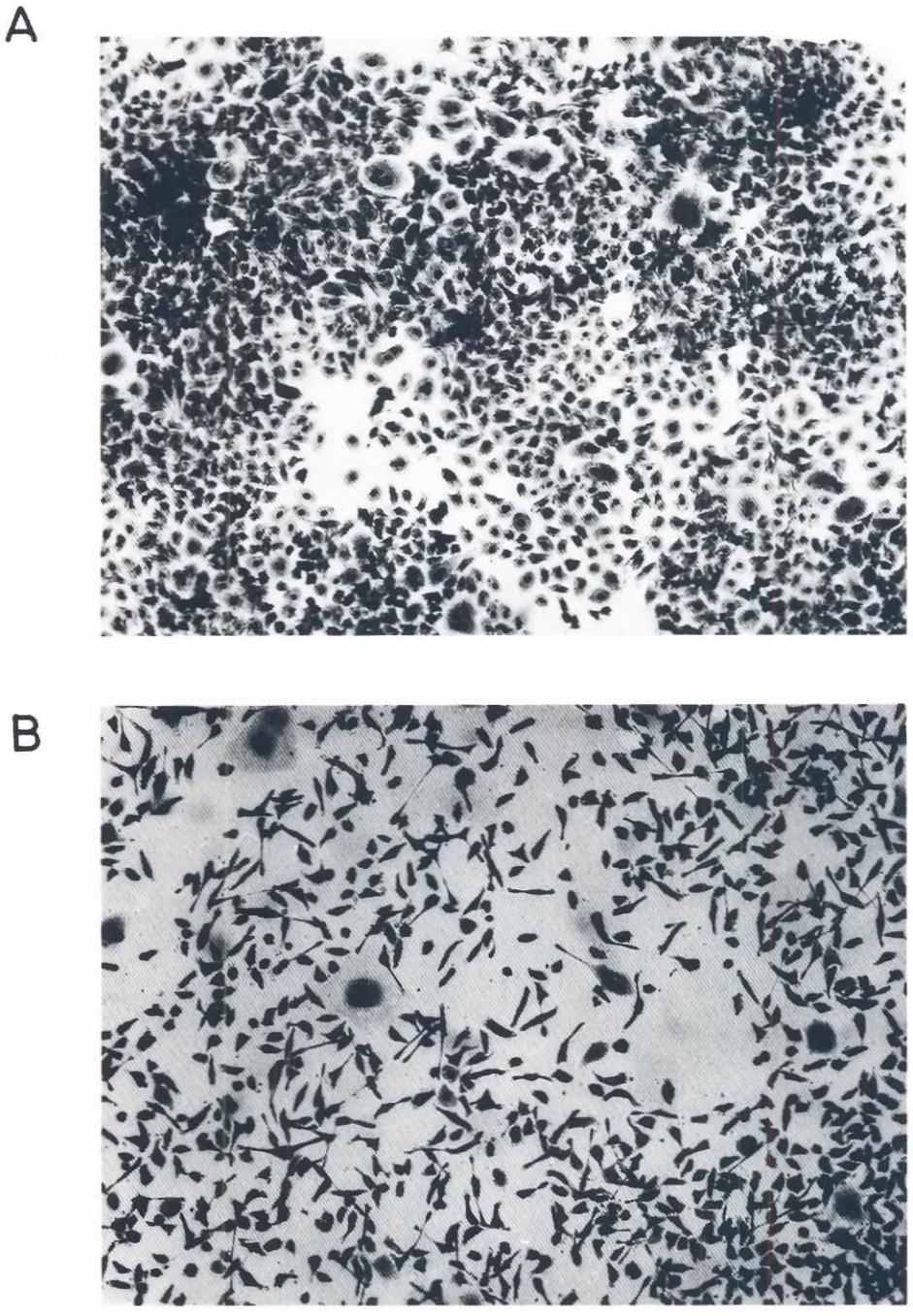

Fig. 3. HEp-2 cells *cultivated in medium supplemented with fetal calf serum* **(A)** *or with normal mouse serum* **(B)**, *respectively.*

combined with 10% CS also suppressed L929 cell growth *in vitro*. The effect was less pronounced if NMLE was used ($P<0.05$).

The growth of HEp-2 cells in the presence of NMS was slightly, but significantly ($P<0.05$), suppressed in comparison to growth abilities in the presence of fetal calf serum (FCS) as presented in Fig. 2. However, the sera from operated mice, *i.e.* either SHMS or PHMS, stimulated HEp-2 cell growth in comparison to FCS ($P<0.05$). Various liver extracts did not influence HEp-2 cell growth, *i.e.* it was the same as observed for the medium when saline was added. It should be noted that HEp-2 cells growing in FCS supplemented medium were characterized by their typical epithelial morphology. However, if any murine sera was used instead of FCS the morphology was completely different, *i.e.* the cells were not so flattened and gave the impression of fibroid-like cells (Fig. 3). This phenomenon was not observed if these cells were cultivated in any serum-free medium used.

Murine fetal cell growth was more stimulated in the presence of FCS ($P<0.05$), than if NMS was used (Fig. 4). Cell numbers in the cultures were even smaller if the sera from operated mice (SHMS or PHMS, respectively) were used ($P<0.05$). On the other hand, liver

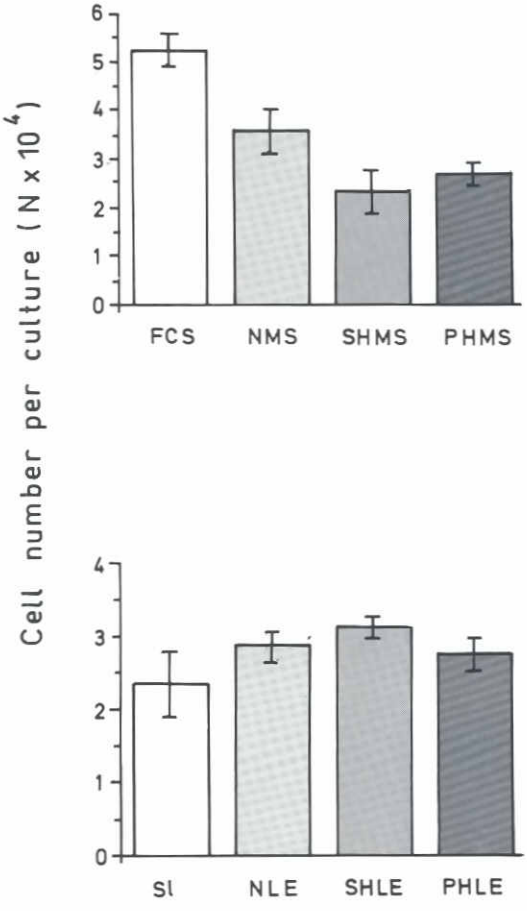

Fig. 4. Numbers of murine fetal cells following cultivation *with fetal calf serum (FCS), normal mouse serum (NMS), sham-hepatectomized mouse serum (SHMS), partially hepatectomized mouse serum (PHMS), normal mouse liver extract (NMLE), sham-hepatectomized mouse liver extract (SHLE), partially hepatectomized mouse liver extract (PHLE), or in medium supplemented with an adequate volume of saline (Sl), respectively.*

extracts did not show any influence on fetal cell growth in comparison to the values obtained for serum-free media (Fig. 4).

Primary cell cultures were performed from melanoma B16 tumor growing in C57Bl/GoZgr mouse hind limb, intact liver or regenerating liver, respectively. The cultures originating from melanoma transplanted in liver grew much better than those obtained from the tumor transplanted in the limb if the media were supplemented with 10% FCS. (Fig. 5). Similar differences were observed if tumor cells were cultivated in the presence of NMS or without any sera in the medium (Fig. 5). Further, as presented, *in vitro* growth of melanoma cells isolated from the tumor growing in regenerating liver was suppressed in comparison to the cells isolated from melanoma growing in intact liver (Fig. 5). FCS was more convenient than any MS for melanoma cell growth *in vitro*. However, there was no significant difference in the growth of melanoma cells originating from the tumor transplanted in the limb if the cells were cultured in any of the murine sera used (Fig. 5). NMS stimulated the growth of cells isolated from the tumor transplanted in intact liver better than the sera from operated mice, while PHMS was the best of all murine sera used for the cultivation of melanoma cells from the tumor growing in regenerating liver (Fig. 5). The different growth features of melanoma cells, due to the different transplantation procedures *in vivo*, could also be observed if the cells were incubated in serum-free medium. There were no significant differences in cell growth abilities if different LE were added to the medium or if the cells were incubated in medium only (Fig. 5). Different LE added to the medium comprising 10% FCS inhibited equally well the growth of melanoma cells isolated from the tumor transplanted in regenerating liver (in comparison to the cultures without LE, *i.e.* with 10% FCS only). However, NLE even stimulated the growth of cells isolated from

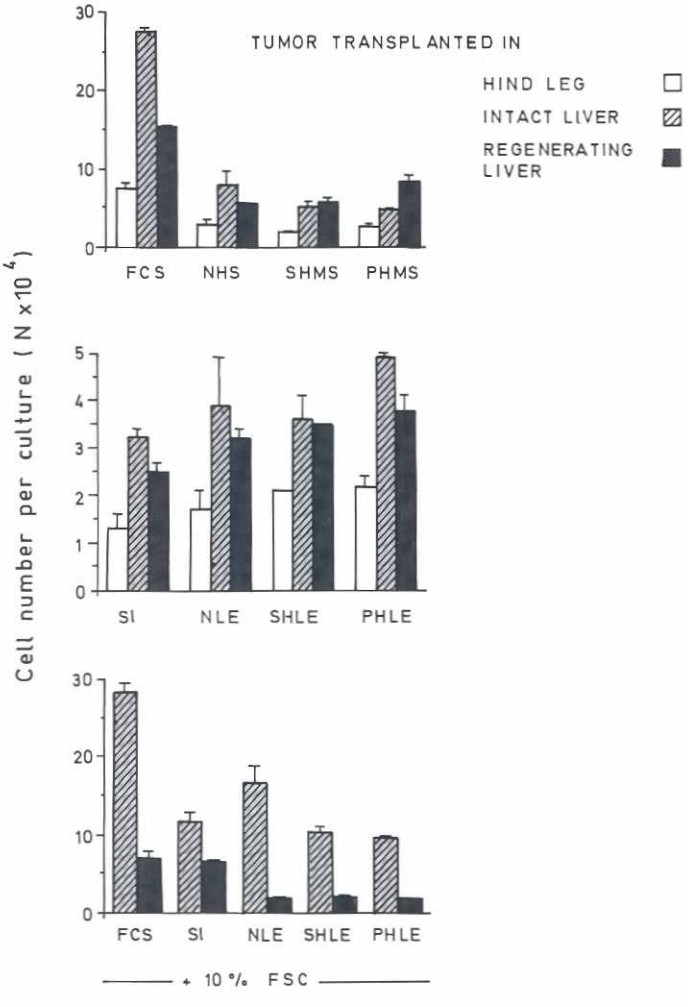

Fig. 5. Numbers of melanoma B 16 cells following cultivation *with fetal calf serum (FCS), normal mouse serum (NMS), sham-hepatectomized mouse serum (SHMS), partially hepatectomized mouse serum (PHMS), normal mouse liver extract (NMLE), sham-hepatectomized liver extract (SHLE), partially hepatectomized liver extract (PHLE), a combination of sera and liver extracts, or in medium supplemented with an adequate volume of saline (Sl), respectively. Melanoma cells were obtained from the tumor transplanted in the mouse hind leg, intact liver, or the liver regenerating after partial hepatectomy, respectively.*

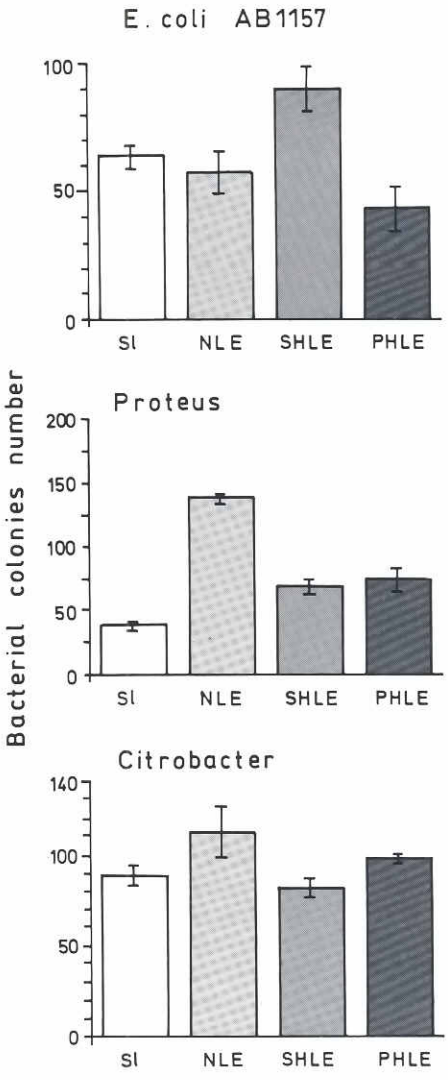

Fig. 6. Numbers of particular bacterial colonies per plate following plating *with the extracts from normal mouse liver (NLE), sham-hepatectomized mouse liver extract (SHLE), partially hepatectomized mouse liver extract (PHLE), or in medium supplemented with an adequate volume of saline (SI), respectively.*

melanoma transplanted in intact liver in comparison to the cultures without LE. Further, there was no difference in the growth abilities of these cells if there was no LE in the culture medium, or if either SHLE or PHLE were added, respectively (Fig. 5).

The influence of liver extracts (LE) on the plating efficiency of bacteria

The plating efficiency of E. coli AB 1157, Proteus, or Citrobacter, exposed to particular LE, was determined. The results were compared to those obtained without LE and are presented in Fig. 6. NLE did not influence the plating efficiency of E. coli, SHLE stimulated it and PHLE inhibited it. Further, all LE significantly ($P<0.05$) stimulated the plating efficiency of Proteus and the influence of NLE was the most prominent (Fig. 6). In comparison to the control the plating efficiency of Citrobacter was about the same if LE was added. However, SHLE in comparison to NLE inhibited ($P<0.05$) the plating efficiency of Citrobacter (Fig. 6).

The modulation of bacterial growth in liquid medium

The medium for Proteus or Citrobacter cultivation was supplemented with different concentrations of used media, *i.e.* the media in which bacteria were growing previously. As presented in Fig. 7 the addition of used medium of Proteus in the concentration of 10% or 50% significantly stimulated the growth of Proteus cultures. Used medium of Citrobacter stimulated Proteus culture growth only if it was present in a 50% concentration (Fig. 7). This stimulation was

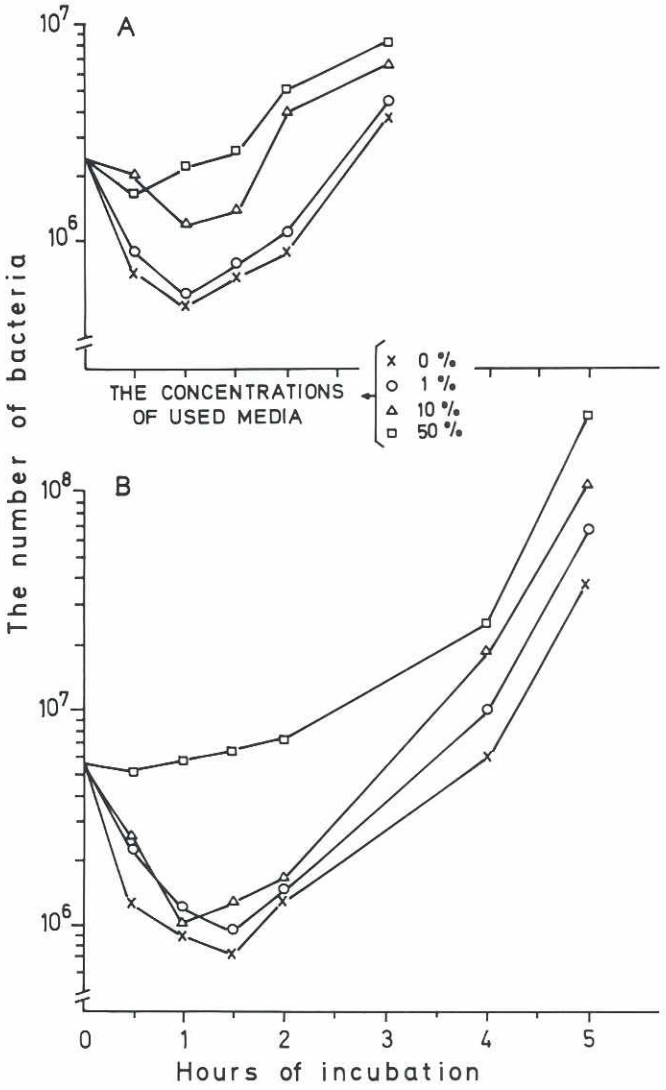

Fig. 7. Number of Proteus strain bacteria in 1 ml of culture following cultivation *in mineral medium (MA) supplemeted with different concentrations of used media of Proteus (A) or Citrobacter (B) strain bacteria, respectively.*

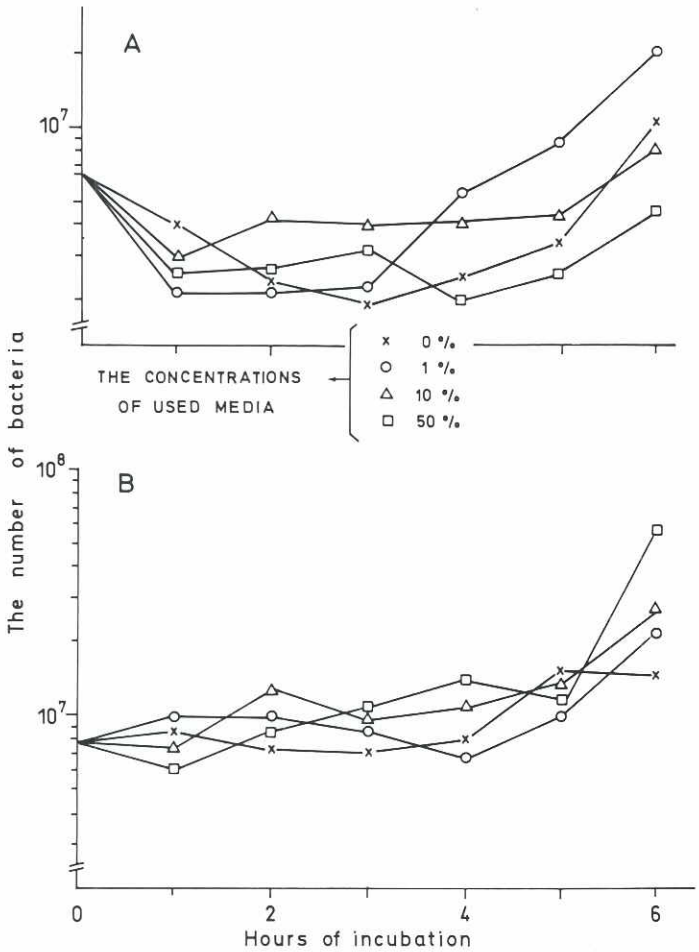

Fig. 8. Number of Citrobacter strain bacteria in 1 ml of culture following cultivation *in mineral medium (MA) supplemented with different concentrations of used media of Proteus (A), or Citrobacter (B) strain bacteria, respectively.*

very prominent during the first hour of cultivation when the number of bacteria significantly decreased in other cultures. However, these used media were not able to modify the growth of Citrobacter (Fig. 8). Further, the growth of Proteus culture was very pronounced if any of the two used media was added in 90% concentrations (Fig. 9). If Citrobacter was cultivated under these conditions a pronounced inhibition of culture growth occurred (Fig. 9). The stimulation of Proteus culture growth, and the inhibition of Citrobacter in the presence of 90% used medium were observed during the first 10 hours of cultivation. Final analysis of the numbers of bacteria was performed after 24 hours for Proteus and after 48 hours for Citrobacter cultivation, respectively. As presented in Fig. 9, at particular times, either Proteus cultures or Citrobacter cultures contained more bacteria than control cultures if they were cultivated from the start with the addition of 90% of used medium.

***The influence of bacterial media, murine sera and liver extracts on murine tumor cell growth* in vitro**

Primary cultures of either melanoma B16 or methylcholanthrene-induced fibrosarcoma were performed from tumors growing in the hind leg of syngeneic mice. The cells were cultivated in Parker's medium supplemented with human AB serum (HS), mouse sera (NMS, SHMS, PHMS), liver extracts (NLE, SHLE, PHLE), or used bacterial media, respectively. The intensity of radioactive thymidine incorporation was determined in these cultures. As presented in Fig. 10, thymidine incorporation was inhibited if murine sera were used instead of human serum ($P<0.05$). Inhibition caused with SHMS was stronger than if other murine sera were used ($P<0.05$). Further, melanoma cells incorporated thymidine equally well with or without HS or if SHLE was added, but the growth was significantly inhibited ($P<0.05$) if NLE or PHLE were added (Fig. 10). Similarly, the addition of either fresh or used bacterial medium did not influence thymidine incorporation in melanoma cells. Fibrosarcoma cells were also sensitive to mouse serum, *i.e.* thymidine incorporation was suppressed in comparison to the cultures with HS. However, HS stimulated incorporation of thymidine in comparison to the cultures without supplements (Fig. 11). NLE did not influence thymidine incorporation, but the extracts from operated mice liver did ($P<0.05$). Furthermore, used bacterial media, particularly those in which Proteus were growing, strongly ($P<0.05$) inhibited thymidine incorporation (Fig. 11).

Discussion

There are several factors incorporated in host-tumor relations that are detrimental for tumor growth. The site where a tumor appears, or, in the experiments, the site where it is transplanted is important for blood formation, possible immunological relations and hormone influences, but certain other conditions in the host tissue (where the tumor is developing) seem to be of further importance. Our particular interest focuses on the influence of regenerating tissue on tumor characteristics. Liver regenerating after partial hepatectomy significantly changed fibrosarcoma anaplasticity and the intensity of pigmentation in melanoma cells (Zarkovic *et al.*, 1985, 1987) indicating that the tumor cells had become less malignant. It is known that the liver regeneration induced by partial hepatectomy is at least in part regulated by humoral factors (Glions, 1956; Weinbren, 1959; Bucher, 1963; Leong *et al.*, 1964; Moolten and Bucher, 1967; Sakai, 1970; Nadal *et al.*, 1976; McMahon and Iype, 1980). Although most of these factors, like hormones or growth factors, are known to be potent growth regulators, they are not specific factors, *i.e.* they do not influence liver regeneration only (Bucher and Swaffield, 1975; Bucher *et al.*, 1978; Farrer *et al.*, 1979; Barbason *et al.*, 1987). However, there are other factors that could be responsible for the systemic control of liver regeneration. Thus hepatocyte growth factor (HGF) was isolated from rat platelets, but its activity was inhibited by adding transforming growth factor ß (TGF ß) *in vitro* (Hayashi and Carr, 1985; Nakamura *et al.*, 1985, 1986 a,b). However, whether HGF is the main factor regulating liver regeneration is still uncertain. Two other factors of different molecular weight higher than 120,000 or lower than 3,000 D, named hepatoproteins A and B (HPTA and HPTB), respectively, have been described as very potent growth factors *in vitro* (Michalopoulos *et al.*, 1984). These factors stimulate preferably the proliferation of cultured liver cells. A slight stimulation of rat skin fibroblast proliferation *in vitro* was also noticed (Michalopoulos *et al.*, 1984).

Whether the mentioned factors could be responsible for the effects of murine sera and liver extracts on cell growth *in vitro*, as presented in this article, remains uncertain. We have observed a

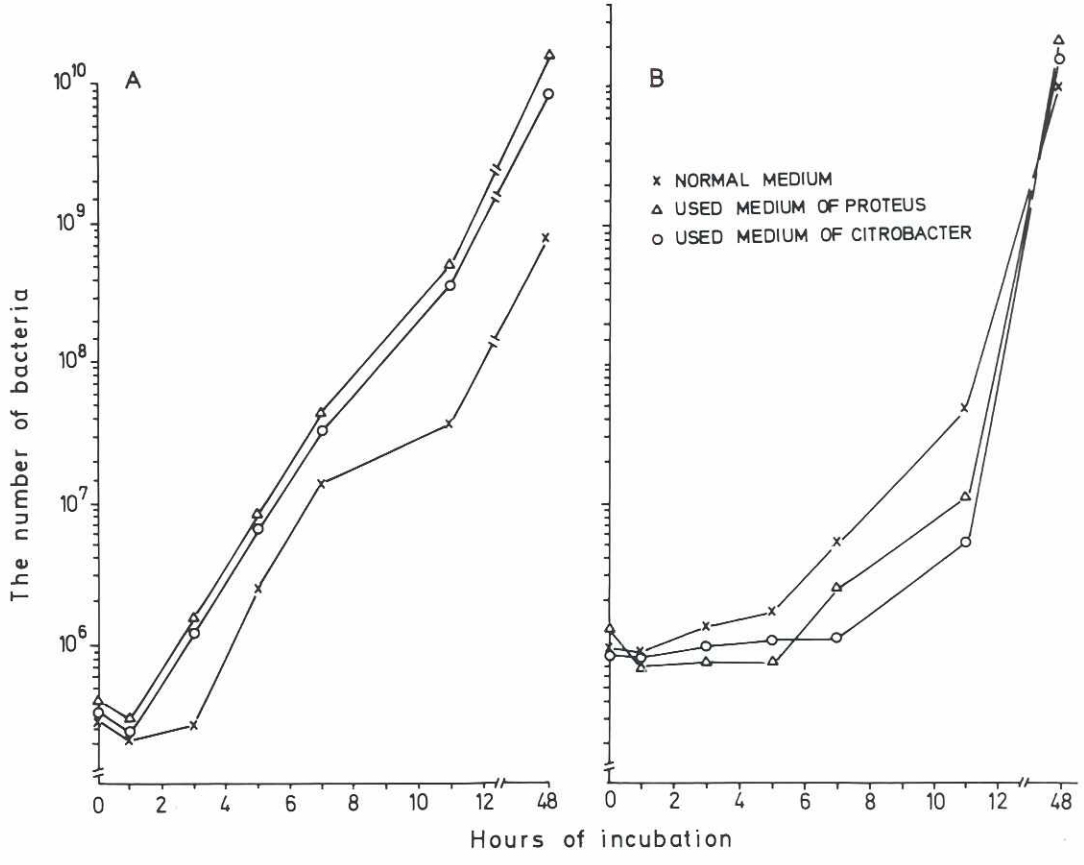

Fig. 9. Numbers of Proteus (A) or Citrobacter (B) strain bacteria in 1 ml of culture following cultivation *in mineral medium (MA) supplemented with 90% of particular used medium, respectively.*

significant influence of the sera and liver extracts used, although they were heated at 56°C for 30 min. It is probable that HPTA is not the main active component of murine sera and liver extracts, while its effect could be significantly decreased after heating the serum at 60°C for 30 min. The presented results indicate that *in vitro* growth of several cell lines or primary tumor cell cultures could be modified by adding liver extracts and/or sera from normal or from operated mice (with or without partial hepatectomy). Liver extracts inhibited the growth of L929 cells but not of HEp-2 cell lines, primary culture of either mouse fetal cells or melanoma cells. Further, the sera from these mice were generally less convenient in comparison to other sera used, except for HEp-2 cells. It should be mentioned that PHMS were more convenient than other mice sera for L929 and for melanoma cells. We cannot explain these specificities, but it is an indication that the phenomenon obtained depends on the model chosen. The possibility that the effects of sera are more pronounced than those of liver extracts is not easy to explain. One can speculate that active components are not synthesized only in the liver, or, if mainly produced there, they are more active after coupling to certain sera proteins. Hence, the obtained results seem to be similar to the effects of the rat serum glycopeptide described by Nadal (1987). The inhibitory effects of serum glycopeptide on hepatocyte proliferation *in vivo* could be abolished by normal adult or baby rat serum, as well as with mouse or human serum, but not with partially hepatectomized or sham-hepatectomized rat serum. It seems that surgical treatment can modify the activity of both stimulatory and inhibitory serum growth factors, and their interactions can influence cell growth. The interaction of serum glycoprotein and acute phase proteins seems very provocative suggesting the possible systemic effects of various disorders (*i.e.* inflammation, malignant tumors, trauma) on the growth capacity of liver cells. Recently, it was described that interleukin 1ß can significantly inhibit adult rat hepatocyte growth *in vitro* and induce production of acute phase proteins in liver cells (Darlington *et al.*, 1986, Nakamura *et al.*, 1988). It also stimulates the proliferation of fibroblasts, but inhibits the growth of human melanoma cells, L929 mouse fibroblasts and mammary carcinoma cells (Schmidt *et al.*, 1982; Onozaki *et al.*, 1985; Gaffny and Tsai, 1986).

Furthermore, there are numerous data indicating the important role of interleukin-6 (IL-6) in the regulation of an organism's response (*i.e.* the acute phase response) to various kinds of «stress», including surgery (Sehgal *et al.*, 1989). Among other effects, IL-6 is known to be a potent multifunctional regulator of growth and differentiation of different cells, including hepatocytes (Sehgal, 1989). Other factors playing an important role in homeostasis are «heat-shock» proteins, which could be responsible for the biological effects of tissue extracts (Currie and White, 1981; Jäättelä *et al.*, 1989). However, their role in liver regeneration is uncertain. On the other hand, ubiquitin, which could be included among heat-shock proteins, modifies the metabolism of proteins

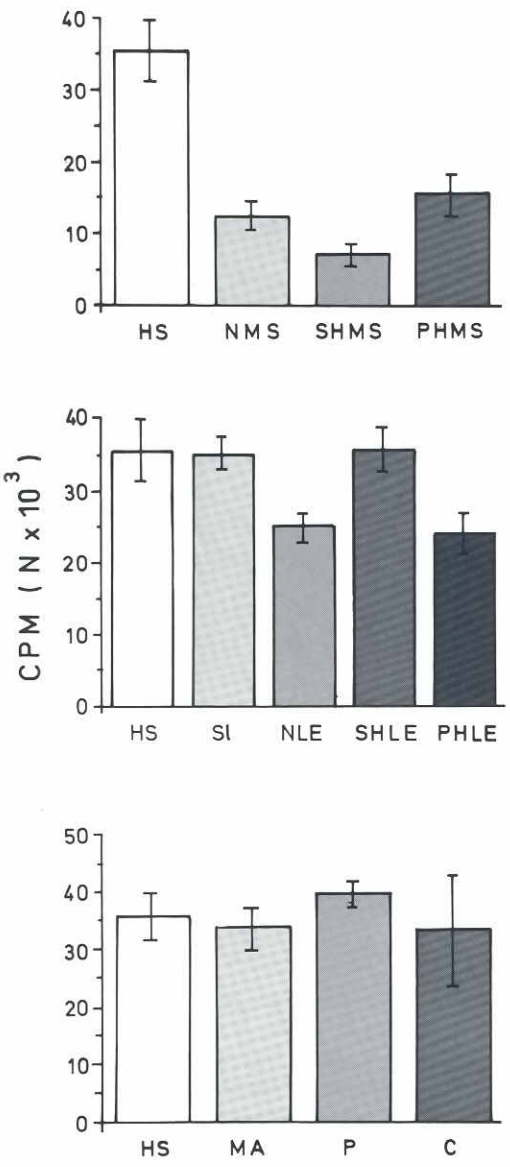

Fig. 10. Radioactive thymidine incorporation rate in melanoma B 16 cells following cultivation *in human AB serum (HS), normal mouse serum (NMS), sham-hepatectomized mouse serum (SHMS), partially hepatectomized mouse serum (PHMS), normal mouse liver extract (NMLE), sham-hepatectomized mouse liver extract (SHLE), partially hepatectomized mouse liver extract (PHLE), medium supplemented with an adequate volume of saline (SI), mineral medium (MA), used media of Proteus (P) or Citrobacter (C) strain bacteria, respectively.*

and determines growth features of the cells (Parag *et al.*, 1987). However, it is not certain that ubiquitin could be a factor regulating tissue regeneration, or, further, that it is responsible for the observed effects of liver extracts on cell growth *in vitro*. Our findings suggest that there are factors that modify liver regeneration but also influence the growth of other cell types. The activity of such regulatory growth factors could be modified by different pathological or maybe physiological processes (*i.e.* tissue regeneration), especially those reflected by acute phase response (Carr, 1983; Kushner, 1988). Hence, our results could be analyzed as another argument for this hypothesis.

The different activity of liver extracts added to the cell cultures incubated in the presence of calf serum or in serum-free conditions is in agreement with the stated hypothesis. The stimulatory effects of one growth factor *in vitro* could be significantly modified by the addition of another growth factor, especially a bifunctional growth factor, such as TGF ß (Todaro *et al.*, 1980; Bowen-Pope *et al.*, 1983; Roberts *et al.*, 1985; Sporn and Roberts, 1985; Carr *et al.*, 1986; Bryckaert *et al.*, 1988). It was also shown that interferon-γ could inhibit the mitogenic activity of different growth factors analyzed on both human and murine fibroblasts *in vitro* (Oleszak, 1988). In contrast, the stimulatory effects of HPTA and HPTB is potentiated in the presence of EGF (Michalopoulos *et al.*, 1984). Thus, the modification of the effects of liver extracts *in vitro* by the presence of calf serum could be explained as the result of simultaneous effects or the interaction between the growth factors present in murine liver extracts and the growth factors from calf serum. The modification in tumor growth characteristics *in vitro*, resulting from different transplantation procedures of melanoma B16, indicate that tumor characteristics change while growing in liver tissue. The stimulation of cell growth *in vitro* was more pronounced if melanoma cells were transplanted into the intact liver, than if they were transplanted into regenerating liver tissue, suggesting that liver regeneration somehow diminished the capability of melanoma B16 cell growth *in vitro*. This result could be explained by the increased sensitivity of melanoma cells to the effects of inhibitory factors probably present in liver tissue. This speculation is mainly based on the observation obtained with tumor cells cultured in medium supplemented with 10% fetal calf serum and 10% liver extracts. It is also in agreement with tumor growth inhibition observed during liver regeneration (Zarkovic *et al.*, 1990). Moreover, it could mean that the simultaneous presence of liver tissue factors and serum factors is necessary to obtain the controlled proliferation of melanoma cells *in vitro*. A similar phenomenon of modulation of *in vitro* growth was obtained by adding liver extracts to bacterial cultures. All liver extracts stimulated Proteus plating efficiency and did not influence Citrobacter abilities. NLE was more stimulative than the others for Proteus. Further, used bacterial media, regardless of origin, stimulated the growth of Proteus more if the concentration increased, but inhibited Citrobacter in high concentrations. Supernatants of Proteus cultures were able to inhibit the incorporation of radioactive thymidine in fibrosarcoma but not in melanoma cells cultured *in vitro*. This might indicate that the factors regulating cell mitosis and the increase in cell numbers are present even in bacteria. Whether it means that one or several common regulators exist in nature appears as a provocative, but still completely open question. However, the data indicating the importance of factors of bacterial origin in influencing the growth of liver tissue *in vivo* point in this direction (Schulte Herman *et al.*, 1976). The expression of protooncogene *ras* during liver regeneration suggests that controlled proliferation of liver cells during regeneration probably results from the autocrine control and the influence of humoral factors (Goyette *et al.*, 1983). Whether the exposure of melanoma cells to the processes controlling liver regeneration induced the programmed cell death (apoptosis) (Kerr *et al.*, 1972, Bursch *et al.*, 1984; Sarraf and Bowen, 1988) in tumorous tissue, thus changing its sensitivity to growth regulation factors when tumor cells were exposed to liver extracts and fetal calf serum *in vitro*, remains unsolved. Wells and Miotto (1986) have

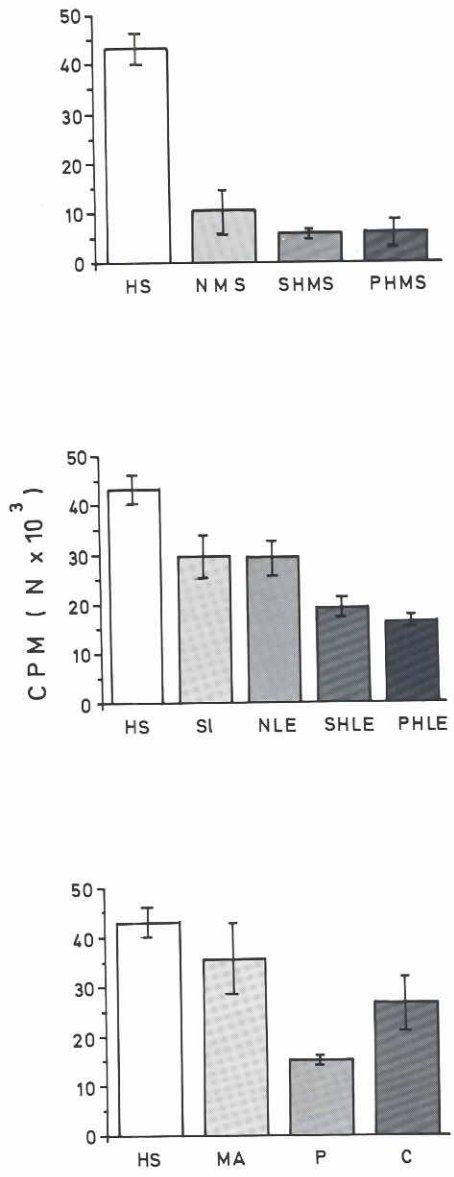

Fig. 11. Radioactive thymidine incorporation rate in methylcholanthrene induced fibrosarcoma cells following cultivation *in human AB serum (HS), normal mouse serum (NMS), sham-hepatectomized mouse serum (SHMS), partially hepatectomized mouse serum (PHMS), normal mouse liver extract (NMLE), sham-hepatectomized mouse liver extract (SHLE), partially hepatectomized mouse liver extract (PHLE), medium supplemented with an adequate volume of saline (SI), mineral medium (MA), used media of Proteus (P) or Citrobacter (C) strain bacteria, respectively.*

shown that neuroblastoma growth was inhibited if tumor cells were injected into the mouse embryo. The inhibition of tumor formation in an 8.5-9.5-day-old mouse embryo could be obtained only if malignant cells were injected in the somite region. The same effect was obtained in 13-17-day-old embryos regardless of the site of tumor cell implantation. What is more, the conditioned media from embryonic limb bud tissue culture slowed down the growth of neuroblastoma cells *in vitro* (Wells and Miotto, 1986). Thus, it is possible that embryonic tissue, adult regenerating tissue and serum contain regulatory growth factors which could be responsible for apoptosis or controlled cell proliferation and differentiation. Numerous factors are involved in the control of regeneration. However, it is not known whether the same or similar factors are also responsible for tumor growth inhibition or for embryonal development. The idea of controlling tumor growth by using such regulatory growth factors is quite intriguing. Unfortunately, the nature of these factors and the mechanisms of their action are not yet sufficiently known.

Materials and Methods

Animals

Forty-five CBA/HZgr male mice (Ruder Boskovic Institute) three months old, weighing 20-22 gr, were used for the preparation of the sera and liver extracts. The animals were maintained in standard conditions with unrestricted access to food and water.

Surgical removal of the medial anterior liver lobe after ligature (30% partial hepatectomy) was performed under sterile conditions according to the method of Higgins and Anderson (Higgins and Anderson, 1931). Partial hepatectomy was performed on fifteen animals, while the remaining 30 mice were either sham-hepatectomized or only anesthetized by i.p. injection of 300 mg/kg Chloralhydrate solution (Kemika, Zagreb).

Preparation of the sera and liver extracts

All animals were sacrificed 72 hours after the treatment. The mice were anesthetized with ether and sacrificed by cutting the blood vessels of the neck. The obtained blood samples were pooled together according to the experimental groups as: nonoperated, sham-hepatectomized and partially hepatectomized mice blood. During the next four hours the blood was kept at room temperature and afterwards centrifuged at 750 G for 15 min. The sera were collected and used for further procedures.

Liver extracts were prepared by pooling together the liver tissue from all the animals according to the experimental groups. Liver tissue was removed from sacrificed donors under sterile conditions and kept in saline solution cooled on ice. Liver tissue was homogenized in saline (1 gr of liver per 15 ml of saline) at room temperature for five minutes using a mixer (Ultra-Turrax, Janke und Kunke K.G).

Liver extracts and sera were further kept in a warm bath at 56°C for 30 min and then centrifuged at $15x10^3$ G for 10 min. Supernatants were separated from the pellet, diluted with sterile saline in a 1:10 ratio, passed through a 0.22µm Millipore filter and frozen. The protein content in murine sera and liver extracts was determined according to the method of Lowry (Lowry *et al.*, 1951).

Cell cultures

In these experiments four different cell cultures were used. Two continuous cell lines – mouse fibroblasts L929 and human laryngeal carcinoma HEp-2 – were grown as monolayer cultures in Eagle's Minimum Essential Medium (MEM), supplemented with 10% calf serum (L929 cells) or fetal calf serum (HEp-2 cells).

Primary melanoma B16 cell cultures were obtained either from tumors transplanted into the hind leg, or from those transplanted into intact or operated (partial hepatectomy) liver of C57Bl/GoZgr mice. The tumors were taken from donors (three animals in each group), bearing the fifth generation of differently transplanted tumors, which were sacrificed on the 14th day after tumor cell injection. Tumor tissues were pooled together from all donors according to group, and cut into small pieces under sterile conditions. The samples were trypsinized three times with 1% bovine pancreas trypsin solution for 10 min at 37°C. After each trypsinization RPMI 1640 medium containing 10% fetal calf serum (FCS) was added. Following the last trypsinization the cells were centrifuged for 10 min at 250 G. The pellet was

resuspended in RPMI 1640 medium supplemented with 10% FCS, and the cells plated.

The same procedure as for the preparation of the cell suspension was used for C_3H murine fetal cells, obtained from 15-day-old-embryos, sacrificed by ether. The cells were seeded *in vitro* in RPMI 1640 medium supplemented with 10% FCS.

L929 and HEp-2 cells were plated in Ø 35 mm Petri dishes at $2x10^4$ concentration, while melanoma B16 and fetal cells were seeded at $4x10^4$ concentration, and incubated overnight. The next day, the samples were washed twice with sterile phosphate buffer and divided according to treatment into different groups. The cultures from the first group were incubated in the medium containing 10% serum from nonoperated, sham-hepatectomized or partially hepatectomized animals. The samples from the second group received medium containing 10% of liver extracts obtained from nonoperated, sham-hepatectomized or partially hepatectomized mice. The third group of cultures comprised those incubated in the presence of 10% of various liver extracts in the medium supplemented with 10% calf serum (L929 cells) or 10% FCS (melanoma B16). Simultaneously, two controls were set. Initially the cells were cultured in medium under serum-free conditions, and the serum was then substituted with an adequate volume of sterile saline. The cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37°C for the following five days, after which the cells were trypsinized and counted. Each experimental and control group of cultures comprised four equally treated samples.

Melanoma B16 cells, as well as methylcholanthrene-induced murine fibrosarcoma CMC1 cells, were also used for the analysis of the intensity of ^{3}H-thymidine incorporation. Tumor cell suspensions were prepared as already described and seeded (10^5 cells per culture) into microcytoplates in the presence of Parker's medium supplemented with 10% human AB serum. According to the experimental procedure the cells were cultured in the presence of murine sera or liver extracts or in the presence of mineral media used previously for the incubation of enterobacteria. Before the murine sera and liver extracts, as well as media of bacterial cultures, were used in the experiment the protein content in the samples was determined according to the method of Lowry (Lowry *et al.*, 1951), and adjusted by dilution with sterile saline. The protein concentration of bacterial media and liver extracts was 35 µg/ml and of murine sera 3.5 mg/ml. After 1 hour preincubation, ^{3}H-Thymidine (methyl-^{3}H-Thymidine, 25 Ci/mmol, Amersham) diluted with medium at a 1:25 ratio was added and the cells cultured in humidified atmosphere containing 5% CO_2, for the following 24 hours. After that, the cells were washed over a filter and the radioactivity of incorporated ^{3}H-TdR was detected in a beta counter (Beckman LS 100C). Each group of cultures comprised four samples.

Bacterial cultures

Three different strains of enterobacteria were used for these experiments: standard laboratory strain of E. coli AB1157, and native strains of Proteus and Citrobacter strains (obtained from the Institute of Microbiology, Zagreb). Bacteria were cultured at 30°C for 24 hours in liquid Trypton broth with aeration, after which 50 µl samples of each strain culture were diluted in sterile saline up to $1:10^7$ dilution. The last dilution of bacteria suspension was at a 1:10 ratio, either in saline or in nonoperated, sham-hepatectomized or partially hepatectomized mice liver extracts. Thus, the obtained suspensions were poured onto agar gel plates and cultured in normal atmosphere at 30°C for the following 24 hours, when the number of developed colonies indicating the plating efficiency of bacteria in the presence of various liver extracts or saline was counted. There were three samples in each experimental group depending on the strain of bacteria and the type of liver extract used.

Bacteria of the Citrobacter and Proteus strain were also cultured in neutral (pH 7) mineral MA medium containing:
$(NH_4)_2SO_4$, 1.0 g, K_2HPO_4, 10.0 g, KH_2PO_4, 4.5 g, Na citrate x 5 H_2O, 0.5 g, $MgSO_4$ x 7 H_2O, 0.1 g, H_2O, 1000.0 ml.

Thus prepared MA medium was supplemented with 0.4% glucose and bacteria were incubated in 50 ml of MA medium for 24 hours at 30°C with aeration. The obtained cultures were centrifuged at 10^4 G for 15 min at room temperature, after which the obtained supernatant was kept at 56°C in warm bath for 30 min, and filtered through a Ø 0.22 µ Millipore filter. The protein content of the media was determined according to the method of Lowry (Lowry *et al.*, 1951). The «used media» were further added at different concentrations to the fresh MA medium in which either Proteus or Citrobacter strain bacteria were cultured at 30°C with aeration for at least 24 hours. Depending on the concentration of «used media» added as a supplement, dilution of fresh MA medium with water was adjusted to achieve equal concentrations of mineral ingredients for all media. At different time intervals after bacteria were seeded in medium, the samples of bacterial cultures were taken, diluted with MA medium and cultured on agar plates, to determine the number of bacteria in the samples, *i.e.*, to analyze the influence of «used media» on bacterial growth in liquid medium.

Statistics

The results were analyzed using the Mann-Whitney test.

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