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STUDIES ON DEVELOPMENT OF EXPERIMENTAL HEPATIC CANCER BY MEANS OF INTRAVITALLY INTRODUCED SODIUM FLUORESCEIN

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Ever since in 1911 WASSERMANN and KEYSSER [37] had observed the affinity of certain dyes to tumours, the specific accumulation of dyes and other substances in malignant tumours has often been studied. This specific affinity is most helpful in the diagnosis, precise localization and therapy of tumours. Although several experiments of this kind have been made with sodium fluorescein [26, 15, 31, 4], the method has not been used to investigate the development and growth of hepatic cancer induced by butter yellow. Such investigations may, however, furnish new data to the morphogenesis of hepatic tumours, and may also assist in deciding the problem whether or not hepatic tumours, precancerous tissues and healthy liver differ in dye storage, or, in other words, whether or not sodium fluorescein has a specific affinity to tumours.

Material and methods

88 white rats, with an average weight of 150 g, were used in the present experiments. 72 animals were treated with butter yellow [each of them receiving a daily dose of 0,008 g of paradimethylaminoazobenzene mixed to the diet, as described in a previous paper (35)], while the rest of the animals served as controls. There are three phases in the development of carcinoma induced by butter yellow, and the test animals were sacrificed accordingly, i. e. the first group from 2 to 6 weeks, the second from 2 to 4, and the third from 6 to 8 months after the beginning of butter yellow treatment. A 1 : 15 dilution of sodium fluorescein was used for intravital staining, in a dose of 10 ml per kg of body weight. The animals were killed 1, 2, 4, 6, 12, 24, 48, 72 144 hours, respectively, after introduction of the dye. Post mortem the liver and kidney were first examined in ultraviolet light and then frozen sections were prepared of these organs. After studying the distribution of the dye under the fluorescence microscope, the sections were stained with Sudan III and haematoxylin-eosin. Some of the animals, both experimental and control, which had not been treated with sodium fluorescein, were used for studying the autofluorescence of the said organs.

Results

The morphological changes observable during the growth of hepatic tumours caused by butter yellow have been described in previous communications [35, 23]. Three phases can be distinguished in the development of the tumour. In the first phase, toxico-degenerative hepatic lesions are observed.

The second, precancerous, phase is characterized by islets of adenomatous proliferating liver cells, as also by focally appearing cholangiofibrosis [29], cholangioadenomas and cholangiocystadenomas. That of the fully grown malignant tumours may be regarded as the third of the above said phases. The various forms of regressive necrobiotic processes could be observed in every phase, such as focal necrosis, desquamation of the glandular epithelium and fatty degeneration, down to utter cellular destruction. No morphological lesions were seen in the kidneys. The parenchyma and stroma of the normal liver generally shows a pale bluish autofluorescence, in agreement with literary data [34, 10, 13, 14]. The endothelial elements give a brilliant yellow fluorescence. The morbid changes induced by butter yellow cause no essential change in autofluorescence either in the proliferating or the degenerating parts of the liver.

As soon as an hour after its introduction, the dye is seen to have intensively accumulated in every lobule. The diffusely stained cytoplasm of the hepatic cells of both the test and control animals is a brilliant green or greenish yellow; the nucleus can be hardly differentiated from the cytoplasm. Damaged areas in the liver of animals treated with butter yellow for 2 to 3 weeks are well outlined by this time ; they give a brilliant green fluorescence. The cellular elements of the somewhat widened Glisson-triangles reveal an orange-red fluorescence, indicating a high degree of dye retention. The endothelium of the sinuses stains like the parenchyma. The connective tissue of the liver, though saturated with the dye, appears both in the experimental animals and the controls in a fainter fluorescence than the surrounding parenchyma. As regards the kidney, its cells stain intensively very soon after the introduction of the dye ; both the upper and lower portions of the nephron, as also the glomeruli and the connective tissue of the vessels display a vivid green fluorescence. In 2, 4 and 6 hours a picture is seen to develop which becomes most pregnant 12 hours after the introduction of the dye. By this time the intact, functioning part of the liver of both the treated and untreated animals has almost entirely excreted the introduced dye (Fig. 1), so that only the autofluorescence of the liver remains conspicuous. Contrarily, the amount of dye accumulated in the degenerated parts (Fig. 2), far from having become less, is not infrequently found to have increased, as substantiated by the appearance of glittering green, yellowish and reddish-green granules beside an intensively green fluorescence. It was found that the granular transformation of the diffuse staining of the cytoplasm becomes more and more pronounced with time.

The kidneys present the characteristic picture of dyeexcretion, with a gradually increasing difference between the kidneys of the animals treated with butter yellow and those of the untreated control group. In the animals, the dye in the cytoplasm of the cells of the proximal convoluted tubules shows a yellowish green, in some areas a reddish, fluorescence, while the glomeruli, the connective tissue and the efferent ducts do not seem to contain any dye. By this time the

tubules of the control kidneys show a vivid green fluorescence only, indicating that some of the dye is being discharged, the greater part of it having been eliminated previously.

Compared with the healthy parts, dye retention in the degenerated areas of the liver becomes still more pronounced after 24 and 48 hours. 144 hours after the introduction of the dye, there is still a considerably high amount of sodium fluorescein, displaying an orange red fluorescence in Glisson's triangles and the cytoplasms of the macrophages around the degenerated necrotic foci. By this time no dye has remained in the kidney of the controls, while the treated animals continue to excrete the dye through the kidney, though at a slower rate.

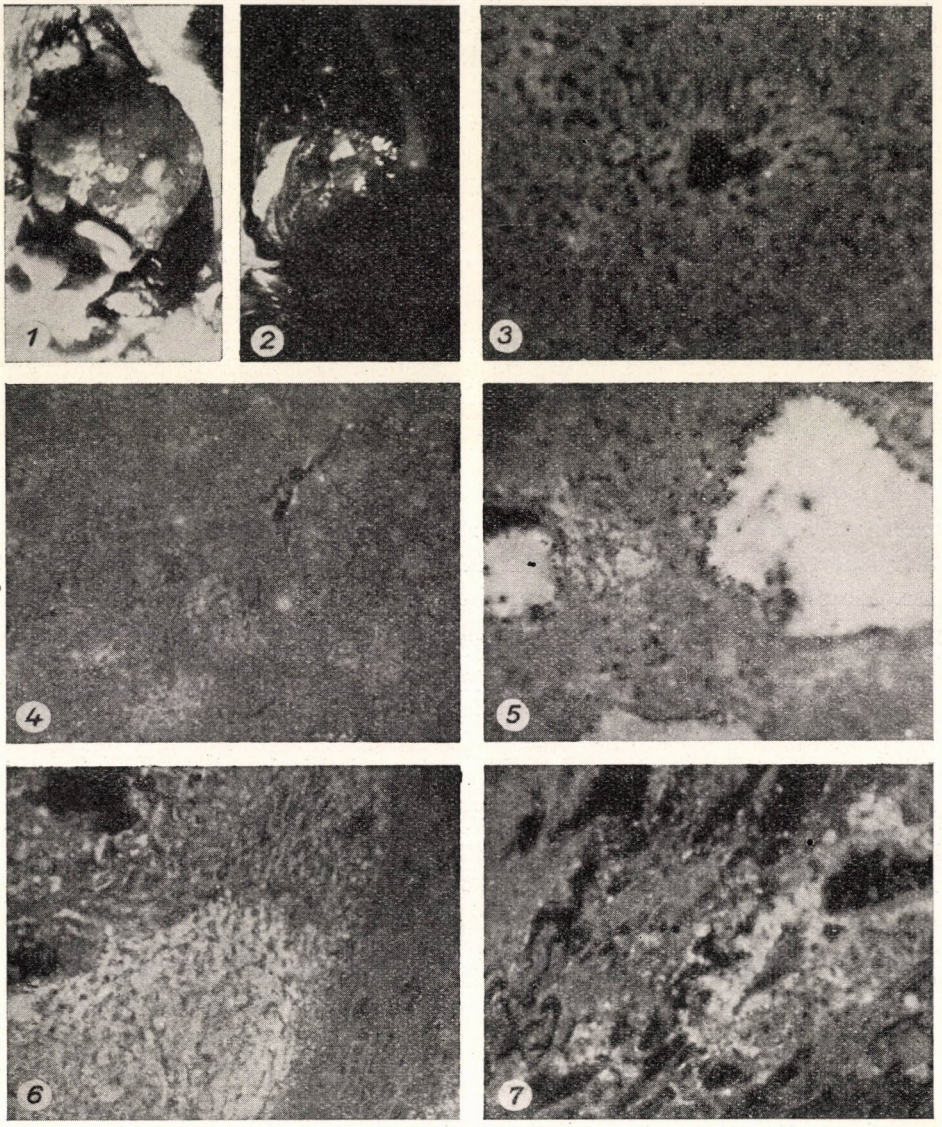
The islets of adenomatous, proliferating, regenerating hepatic parenchyma, as well as the cholangioadenomas and cystadenomas display the same behaviour as the healthy liver. The foci of cholangiocellular adenocarcinoma and hepatocellular carcinoma present a similar picture.

The number of deteriorating elements and degenerated cells is found steadily to increase in the precancerous, and still more in the cancerous, areas. Their fluorescence is similar to that of the toxico-degenerative lesions in the liver. Soon (from 1 to 4 hours) after the dye had been introduced there is still some of it in every cell, but the brilliantly green fluorescence of the contents of the cyst (Fig. 5) and the greenish yellow colour of the degenerating cells are already conspicuous. Somewhat later (6 hours), only a slight greenish blue coloration is seen in the intact epithelial cells in the precancerous and cancerous foci, while necrosing elements are sharply distinguishable by their more intensive yellowish green fluorescence. This difference becomes more conspicuous with time. After the lapse of 144 hours, both the proliferating cancerous tissue and the hepatic parenchyma show autofluorescence only, whereas a high degree of dye retention is still observable in the necrobiotic and necrozed parts (Figs. 6, 7).

The kidney of animals in the precancerous and cancerous states eliminates sodium fluorescein exactly in the same manner as that of the animals that are still in the phase of toxico-degenerative lesions. In this case, too, the dye is excreted slowly. The liver of animals treated with butter yellow was also stained with sodium fluorescein and examined in ultraviolet light. It was observed that at first the intensive greenish yellow fluorescence had been diffuse but later the dye accumulated in foci (Figs. 1, 2). These foci corresponded to the degenerating areas and cysts of the precancerous and cancerous liver. Intensive fluorescence of a similar kind was not observed in any other organ.

Discussion

In former studies concerning the affinity of dyes to tumours mostly semicolloidal acid diazodyes of low diffusibility were used [24, 8, 3, 39, 17, 38, 7]. MOORE et al. were the first to apply sodium fluorescein in man [25, 26, 27].



Figs. 1—2. Hepatic tumours 12 hours after introduction of sodium fluorescein
Fig. 1. Photograph made in white light Fig. 2. The same in ultraviolet light. Cysts and degenerating foci show intense fluorescence. Photograph made under the fluorescence microscope.
Fig. 3. Control liver 12 hours after the administration of sodium fluorescein. Having discharged the greatest part of the dye, the hepatic cells are conspicuous by their autofluorescence only (approximately ×200)
Fig. 4. Liver in the incipient, i. e. toxico-degenerative, phase of treatment with butter yellow (3 weeks), 12 hours after introduction of sodium fluorescein. Increased dye retention by cells in injured zones (approxim. ×90)
Fig. 5. Liver in precancerous state, 6 hours after introduction of sodium fluorescein. A considerable amount of dye is observable in the glandular lumina of the cholangioadenoma. No dye is as yet visible in the surrounding hepatic tissue (×250)

SHAPIRO and LANDING [32] and more recently, CRAMER, BRILMAYER et al. have studied the distribution of various fluorochromatic dyes in experimentally induced tumours [1, 2, 4, 5, 6].

According to JACOBY [16], every kind of change, inflammation or degeneration, etc. would induce a change in the capacity of the tissues to accumulate dyes. Some authors [24, 3, 8, 29] attribute the intensified dye-uptake of tumours to a change in vascular permeability, while, according to MOORE [26], the increased capacity of gliomas to accumulate sodium fluorescein and labelled J^{131} diiodfluorescein may be due to an injury within the tumour of the blood-brain-barrier. KARCZAG et al. were the pioneers in this field [17, 18, 19, 20]; they demonstrated the electrotropism of triphenyl-methane dyes, the affinity of these substances to electronegative tissues; this means at the same time that these dyes are increasedly bound by necrotic elements in the tumours (necrotropism). FIGGE et al. [11, 29] found that porphyrin is stored by lymph-nodes and tissues (tumours) with a high mitotic index.

VEKERDI et al. [35] studied the accumulation in tumours of intravenously injected colloidal polonium by radioautography and found more polonium in the haemorrhagic necrotic parts of Guérin's carcinoma than in its healthy areas. While MOORE denies that sodium fluorescein would accumulate in the necrozed parts of tumours, SHAPIRO and LANDING [32] claim to have found, 7 hours after introduction of the dye, fluorescence only in necrozed areas.

Diachromatic dyes differ from fluoro-chromatic ones not only in that the former can be demonstrated in very low concentrations but also in the higher diffusibility of the diachromatic dyes which enables them to stain every cell in the organism. This property is in some way connected with the rapid excretion of this dye. (RIES 30). Hepatic excretion of sodium fluorescein was studied in amphibians by ELLINGER and HIRT [9], and KULPE [22] and in rats by HANZON [14]; the latter author observed that if small quantities of sodium fluorescein had been introduced into the organism, normal hepatic cells did not excrete the dye by simple diffusion but discharged it into the bile ducts rapidly, within 3 hours, by Hoeber's "active transfer" mechanism.

The present experiments have proved that sodium fluorescein introduced into organs with malignant tumour is retained by the necrotic and necrobiotic parts. It was found that, while during the first hours all intact cells (those in healthy and in tumorous tissue alike) rapidly take up and excrete the dye, damaged cells retain it irrespective of whether they belong to normal parenchyma or

Fig. 6. Hepatocellular carcinoma 24 hours after introduction of sodium fluorescein. The dye is almost completely eliminated from the stroma. Excretion by the degenerative focus visible in the lower part of the picture lags behind the intact areas of the tumour (×250)

Fig. 7. Carcinoma cholangiocellulare adenomatosum. 144 hours after administration of sodium fluorescein the degenerating areas of the tumour and the detached cells in the glandular lumen still contain some of the dye (×250)

tumorous tissue. This phenomenon seems to prove that uninjured, proliferating cells, though belonging to precancerous or cancerous tissue, do not lose their capacity of "actively transferring" sodium fluorescein and that they excrete it quite in the same manner as the cells of the normal hepatic parenchyma ; while all damaged cells become incapable of "active transfer".

The affinity of fluorescein to tumours appears, therefore, to be based on the fact that cancerous parenchyma degenerates more extensively than normal tissue [12, 31].

The present observations are in complete agreement with the findings of MOORE [25, 26], according to which the affinity of the dye to tumour tissue is not really a specific affinity but is attributable to intensified regressive processes. The soundness of this theory was further substantiated by our failure to observe any qualitative difference in dye-retention between precancerous tissues and malignant tumours.

It was demonstrated by MOORE's [25, 26] experiments that 85 per cent of the fluorescein introduced is excreted through the liver and 15 per cent through the kidney. If the bile ducts are occluded, a correspondingly higher rate of the dye is eliminated through the kidney.

Renal excretion is also predominant in animals treated with butter yellow, liver function being disturbed by the dye. The intrahepatic, i. e. intratumoral, accumulation of the dye explains for the simultaneous protraction of renal excretion.

To interpret the intensive dye accumulation correctly, changes in vascular permeability and in internal lymphcirculation of the tissues have also to be taken into account, since these factors too play a decisive role in the distribution of the dye in the organism. This problem forms the subject of a future communication [34].

Summary

The localization of intravitally administered sodium fluorescein in liver carcinoma provoked by treatment with butter yellow has been studied under the fluorescence microscope.

While the dye was rapidly excreted by the uninjured cells of the liver and the proliferating cells in both adenomas and malignant tumours, it was retained for a considerable length of time by necrozed areas. Renal excretion of sodium fluorescein was also protracted in animals treated with butter yellow.

It has been demonstrated that there is no difference between precancerous and cancerous tissue as regards the uptake and excretion of the dye. The intensified retention of sodium fluorescein in hepatic tumours may be attributed to some specific affinity of the dye to tumours but is due to the presence of extensive degenerative necrobiotic foci in tumorous tissue.

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ИССЛЕДОВАНИЕ РАЗВИТИЯ ЭКСПЕРИМЕНТАЛЬНО ВЫЗВАННОГО РАКА ПЕЧЕНИ С ПОМОЩЬЮ ПРИЖИЗНЕННОЙ ОКРАСКИ ФЛУОРЕСЦЕИНОМ НАТРИЯ

Й. ШУГАР и К. ЛАПИШ

Авторы исследовали флуоресценцмикроскопическую локализацию прижизненно введенного флуоресцеина натрия, при вызванном подачей «Буттергельб»-а (p-dimetilamino-arobenzol) раке печени и во время развития последнего.

Красящее вещество быстро выделяется неповрежденными клетками печени, клетками аденом и разрастающими клетками злокачественных опухолей, в то время как вырожденные некротические части задерживают его более длительное время. У крыс, обработанных «Буттергельб»-ом, выделение флуоресцеина через почки протягивается.

Авторы установили, что накопление красящего вещества в преканцерозных и опухолевых печенях или же их отдача красящего вещества происходит одинаковым образом. В основе повышенной задержки флуоресцеина натрия в опухолях печени лежит не какое-нибудь специфическое сродство к пухоли этого вещества, а повышенное количество дегенеративных некробиотических очагов.

UNTERSUCHUNG DER ENTWICKLUNG VON EXPERIMENTELLEM LEBERKREBS MITTELS NATRIUMFLUORESZEIN-VITALFÄRBUNG

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Die Fluoreszenzmikroskopische Lokalisation des vital verabfolgten Natriumfluoreszeins wurde bei Buttergelb, Leberkarzinom, und während der Entwicklung desselben untersucht.

Der Farbstoff wird von den unbeschädigten Leberzellen, den Adenomzellen und den wuchernden Zellen bösartiger Geschwülste rasch ausgeschieden, während die degenerativen nekrotischen Teile den Farbstoff längere Zeit zurückhalten. Bei mit Buttergelb behandelten Ratten wird die Ausscheidung des Natriumfluoreszeins durch die Niere verzögert.

Es wurde festgestellt, dass die Farbstoffspeicherung der präkanzerösen und tumorösen Lebern, bzw. ihre Farbstoffabgabe in gleicher Weise vor sich gehen. Die Zurückhaltung des Natriumfluoreszeins in Lebergeschwülsten ist nicht auf eine spezifische Tumoraffinität des Farbstoffes, sondern auf die erhöhte Anzahl der degenerativen nekrobiotischen Herde zurückzuführen.

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