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COMPARATIVE STUDIES OF NUCLEIC SUBSTANCES IN FLORID AND IN NECROSED TISSUES

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(Received March 29, 1955)

The biological significance of nucleic acids and of nucleoproteins explains the recent increased interest devoted to these substances in tumour research.

Quantitative data concerning nucleic acids in tumour tissue can be found in the works by LEUCHTENBERGER, KLEIN and KLEIN, PETERMAN and SCHNEIDER, W. C. SCHNEIDER, CERECEDO, REDDY et al., VENDRELY et. al., as well as in the reports by KHOUVINE and GRÉGOIRE, the latter being of particular interest in the present study, since the experiments had been made on Guérin tumour, the same growth we have studied.

A number of reports have also dealt with qualitative studies of nucleic acids in tumours (SPIEGEL—ADOLF, WOODHOUSE, TSUBOI, KHOUVINE, etc.).

The data in the literature are conflicting in a number of questions. This is due partly to the fact that these highly polymerized compounds (which are infinite series of smaller components, called oligonucleotides, that branch off in many directions and contain several cross-linkages) are very labile. While they are being studied, the compounds undergo depolymerisation first and re-polymerisation later and thus results obtained are characteristic not of the original nucleic acid, but of an artifact.

Contradictions found in literature originated often of using different kinds of tumours in experiments, and different tumours differ from each other in this respect, too. Finally, it has to be borne in mind that most of the test objects were either homogenisates of whole tumors or isolated nuclei of cells and no distinction was made between the florid and necrotic parts of a given tumour.

Necroses arising in rapidly growing tumours have been found to have an influence upon the further development of the tumour and these regressive processes have their role also in the formation of metastases.

These findings had stimulated us to study in detail the nucleic acids in the necrosed parts of tumours.

Only very few data are available in the literature concerning the nucleic acid content of necrosed tumour parts. CERECEDO and REDDY have reported that in the florid parts of mouse sarcoma and of spontaneous mammary cancer

the amount of ribonucleic acid (RNA) was decreased, while in necrosed areas both RNA and DNA (desoxyribonucleic acid) were decreased.

Accoriding to KHOUVINE and GRÉGOIRE, necrosis is characterised by a disappearance of RNA ; they have found that the level of RNA in necrosed areas of the Guérin tumour was 1/3 of that found in non-necrosed parts of the tumour.

Also CRABB and KELSALL have stated that in liquified necrosed areas RNA cannot be detected by histochemical methods, while in the same areas DNA could be detected by means of the Feulgen reaction.

Experimental

Guérin's transplanted rat tumour was used as the test object. The animals were killed by bleeding at about 2 months following transplantation. The tumour grew large in every case and its inside was completely necrosed. The necrotic mass inside the tumour was separated from the shell-like external coat of tumour tissue as completely as possible. The two parts were homogenized separately and in each of them the nucleic acid content was estimated, always in four parallel determinations. The different nucleic substances were separated by the method of SCHMIDT and TANNHÄUSER and the phosphorus content of the single fractions was also determined. The ratio between the single components was then calculated from a comparison of these P values.

We have modified the Schmidt—Tannhauser method in the following way. DNA was calculated not only indirectly, but after acid precipitation the DNA precipitate had been redissolved and its phosphorus content was directly measured.

Five tumours were examined in this way, i. e. the total number of tests was 40.

Results

Histochemical studies by the Feulgen technique have shown that in necrotic areas nuclei disintegrate into granules and nuclear substance disappears in the central areas of necrosed parts. It was therefore anticipated that a considerable decrease in the quantity of DNA would in this way ensue. In contrast with this, quantitative analysis showed DNA to be present in unchanged amounts in both florid and necrosed areas, while the quantity of RNA was reduced significantly in every case. As seen in Fig. 1., the rate of decrease was not uniform : it probably depends upon the rate of progression and the extent of necrosis.

In Fig. 1 each column represents the mean of 4 parallel tests and the five pairs of columns show the RNA and DNA, respectively, contained in 5

different tumours ; the shaded column indicates the florid part, while the unshaded one the necrosed part of the same tumour.

As it has been mentioned above and as it is clearly visible in Fig. 1, the decrease in RNA was very considerable (22 to 43,5 per cent of the RNA in the florid part). The necrosed area contained slightly more DNA in 3 cases, and

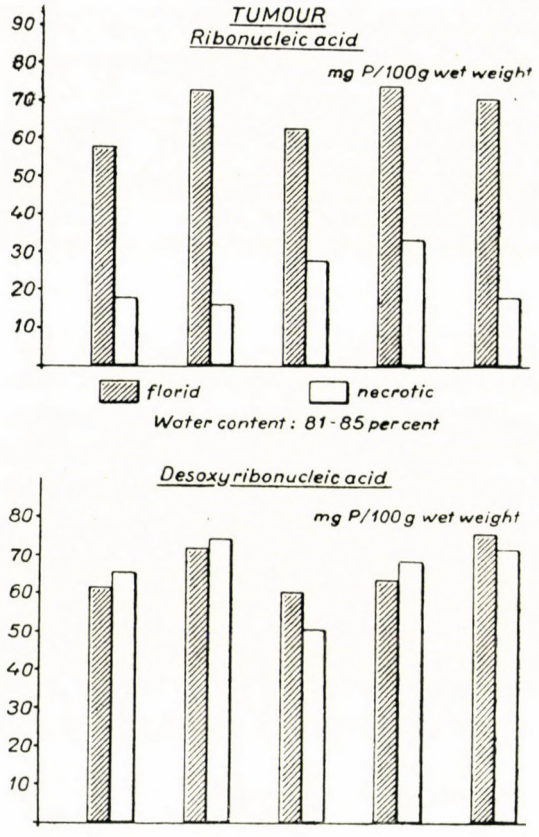

Fig. 1

slightly less in 2 cases, than did the florid part. The divergence varied between 3 to 7 per cent in four cases and a difference as high as 17 per cent was detectable in one case only. The results being within the limits of experimental error the conclusion may be drawn that florid and necrosed areas contain approximately equal amounts of DNA.

In Table I. are presented the numerical values, to facilitate the comparison of parallel tests.

It can be seen that the deviation among the results was $\pm 4-12$ per cent.

Our results for DNA do not agree with the results reported by CERECEDO

Table I

No	R. N. A. Intact parallel 1	2	3	4	Mean	Deviation ± %	Necrotic parallel 1	2	3	4	Mean	Deviation ± %
1.	54,5	59,2	62,8	51,8	57	10	18,7	19,5	17,3	16,9	18	8
2.	77,4	73,0	66,0	71,5	72	8	14,8	15,7	17,4	16,0	16	9
3.	59,3	54,6	66,5	68,8	62	12	25,1	27,3	31,0	28,6	28	10,5
4.	71,2	64,0	82,1	74,7	73	12	36,6	29,3	30,2	36,0	33	11
5.	69,0	68,1	73,2	70,2	70	4,5	19,8	16,3	16,7	19,5	18	10
	D. N. A. Intact parallel 1	2	3	4	Mean	Deviation ± %	Necrotic parallel 1	2	3	4	Mean	Deviation ± %
	57,7	59,4	65,2	65,8	62	7	63,4	65,7	69,1	65,8	66	4,5
	66,4	68,3	77,0	76,3	72	7,5	69,3	71,1	77,1	77,9	74	6,3
	54,1	58,3	63,9	63,7	60	10	48,0	53,1	49,2	47,7	50	6
	58,5	59,6	69,7	68,2	64	9	72,1	70,4	66,3	67,2	69	4,5
	74,8	77,3	79,1	72,8	76	4,2	74,4	73,7	69,1	70,8	72	4

and REDDY, who found a decrease in DNA in the necrosed areas of mouse sarcoma and of spontaneous mammary cancer.

In connection with the disappearance of RNA, numerous problems have emerged. First of all, we had to find out what happens to RNA? It is likely that

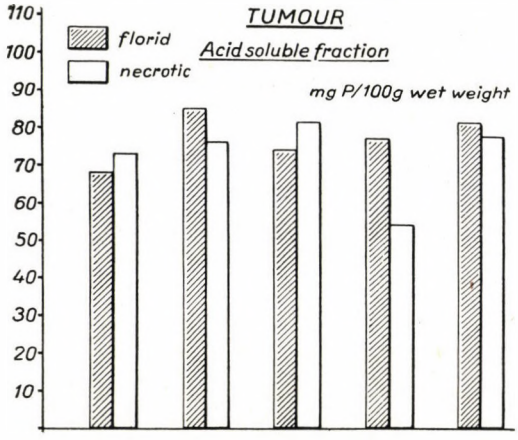

Fig. 2

it is broken down to nucleotides, or, still further, to inorganic phophates, purine and pyrimidine bases. And even then it is questionable whether these depolymerised breakdown products remain in the necrosed parts of the tumour or are removed.

The data in Fig. 2. indicate that these substances do not remain in the necrosed areas of the tumour, at least as far as one can judge from the phosphorus values; this is clear from a comparison of the acid-soluble fractions in the intact and the necrotic parts of the tumour. The acid-soluble fraction contains namely the mono-nucleotides, the inorganic phosphates and the other phosphor-containing, acid-soluble breakdown products of nucleic acids. The

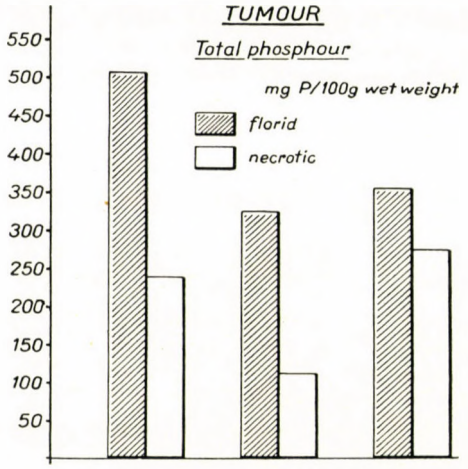

Fig. 3

data in Fig. 2. obviously show that necrosed tumour-parts do not contain more acid-soluble parts than intact parts.

We had to exlude also the possibility that the missing amount of P (*i. e.* some part of RNA) had been dissolved in some way by the lipid solvents used, together with the phospholipids. In order to elucidate this point, intact and necrosed parts from three tumours were examined for their total P content. The results of these estimations are shown in Fig. 3.

The results in Fig. 3. show that the total amount of P is also decreased in necrosed areas, so that the amount of phosphor-containing lipids could not increase. From this it follows that RNA had been removed from the necrosed parts of the tumour.

The disappearance of RNA might be explained in two ways. RNA or its breakdown products may leave the tumour tissue proper, which would indicate that even so-called necrosed tissues have a metabolism. The other, and more probable, explanation is that the breakdown products are utilized by the florid,

viable parts of the tumour for building up from them their own nucleic substances. In support of the latter theory appear to be the findings by CRABB and KELSALL, who found by histochemical methods that a zone showing markedly increased basophilia, 3 to 6 cells in width, was detectable in areas adjacent to necrosed parts. Similar observations have been made in surviving liver tissue cultures by TÖRŐ, PÓSALAKY and BARKA, of the Budapest Institute of Embryology and

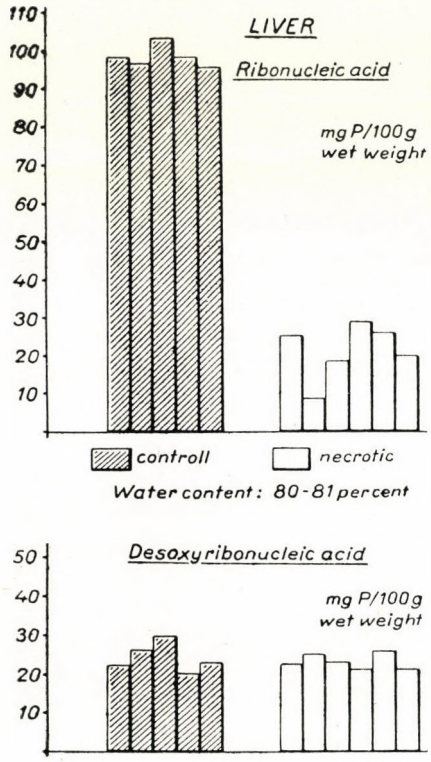

Fig. 4

Histology. The latter authors have reported that at the margin of the central necrosing areas an increased pyroninophilia was demonstrable, indicative of an increased RNA content.

Further studies should determine why it is just the RNA content that decreases in necrosed areas. It seems probable that the high stability of DNA and the very labile nature of RNA are factors to be considered in this respect.

The next problem to be solved was whether other types of necrosis would yield the same results. In an effort to elucidate this point, necrosis was induced in 1 or 2 lobes of rat livers ligating the hilar vessels leading to them. The animals were killed by bleeding 6, 24, 48, 96, 144, and 240 hours following the operation,

respectively. When ligation had been imperfect, the capsule became thickened and regeneration started. There ensued, however, centrilobular necrosis even in such cases. When ligation had been succesful, the lobe as a whole fell victim to necrosis.

After detaching the thickened capsule from the necrosed part, the necrosed part, the intact hepatic lobes, and the thickened capsule were tested for their

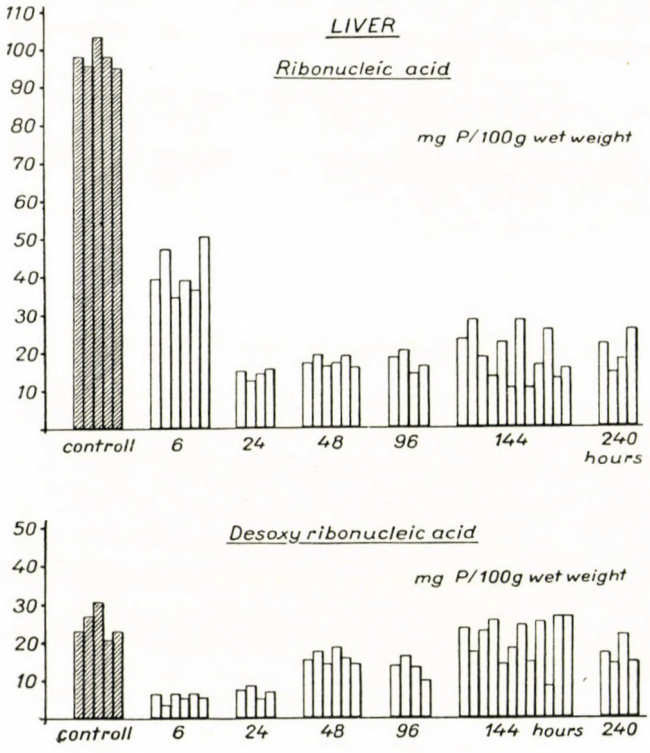

Fig. 5

content in nucleic acid, making 4 parallel estimations of each specimen as it had been done with tumours.

This series consisted of 36 experiments involving a total of more than 160 estimations. The livers from 5 animals served as control. The results yielded by the control livers were identical with those obtained for intact lobes from operated animals and both were in agreement with the values published in the literature.

Fig. 4 is a comparative presentation of the nucleic acid values in necrosed and in control livers (Fig. 4).

The necrosed parts contained the same amount of DNA as the control livers, while RNA was markedly decreased in the former, like in tumours.

Fig. 5 shows the time relations of the changes in nucleic acid content, as they took place in necrosis of the liver.

It can be seen that after six hours the reduction in the amount of RNA is already as high as 50 per cent, and 24 hours following the operation the RNA content of the necrosed area falls to 15 to 20 per cent of the initial, the DNA content, too, shows a considerable decrease initially, but 144 hours after operation it is again comparable to the amount present in normal liver tissue. (In 7 cases the values ranged from 20 to 30 mg per 100 g, as in the controls, while in 2 cases values of 17 mg per 100 g, in another 2 of 14 mg per 100 g, and in 1 case less than 10 mg per 100 g were obtained.)

It has been mentioned that the subcapsular granulation tissue was also examined. Like in every kind of proliferating tissue, in this case, too, the DNA content was higher than in the normal liver. As to RNA values, these were lower than normal, but higher than in necrosed liver tissue.

Histological

Guérin tumour. This type of tumour was especially suitable for our studies, because it contains necrotic foci readly distinguishable by gross examination right after it has become palpable. The tumours used in the experiments weighed from 50 to 80 g, a weight the tumour usually attains in 2 months. The main mass of florid, intact parts was located at the periphery of the tumour, subcapsularly, and was 1/2 to 3 cm thick. Most of the tumour consisted of a central necrosed mass, yellowish-grey in colour, friable, caseous, with map-like outlines. In some cases bloody inhibition also occured.

The sections prepared from tumour tissue were stained with haematoxylin and eosin (HE), Feulgen's stain or methylgreen-pyronin. The necrobiotic and necrotic areas had rather distinct outlines and exhibited various morphological signs of destruction.

KELLNER has distinguished beween four types of regressive morphological changes :

i. Intact, unaffected tumour cells,

ii. Dissociated cells, with loosened cellular bondage,

iii. Completely dissociated cells showing extensive pyknosis,

iv. The zone composed of totally destroyed cells, in which the nuclei do not stain, but cell contours are still detectable and nuclear detriment, chromatin globules are present.

By separating though only mechanically necrotic areas from intact parts, we have obtained for chemical estimation a mass containing essentially pyknotic and necrotic cells.

In the sections stained by Feulgen's method the zone of total necrosis just stained a light pink or not at all. There was, however, a rather wide margin

adjacent to necrotic areas which consisted of nuclear detriment and pyknotic nuclei. This zone, which gave an intensive DNA reaction, was actaully a necrosed area, in which nuclear detriment had accumulated, along with some leucocytes and macrophages.

Essentially the same pattern was seen in preparations stained with methylgreen and pyronin. Pyroninophilia was very slight in the marginal zone of the necrosed area. The cells and the cellular detriment, however, stained a very intensive green, as a sign of increased amounts of DNA.

Both pyroninophilia and uptake of methylgreen were very scarce in the internal zone of the necrosed area, while the normal areas stained in a regular fashion.

Liver. 24 hours after operation the ligated lobes of the liver had a smooth surface and were mildly swollen. Their surface and cut surface were homogeneous and of a pale yellow colour. After 48 hours a fine fibrinous attachment to normal lobes, peritoneum and intestines was already observed to be present and later to increase along with the progression of necrosis.

In sections made from totally necrosed hepatic lobes, 3 principle zones could be observed. There was an area exhibiting coagulation necrosis, adjacent to it a zone loaded with chromatin granules and, finally, 48 hours following operation a granulation tissue proliferation of the capsule in some cases.

The pyknotic zone found at the periphery of the necrosed area was detectable not only at the periphery of the lobe, but also in areas adjacent to single major foci of necrosis.

In the area of 24-hour necrosis the Feulgen test was mildly positive in but a very few cells (Fig. 6), while in the marginal zone the positivity of the reaction was very obvious.

The pattern seen after staining with methylgreen and pyronin was essentially similar. The accumulations of chromatin in the marginal zone took the dye especially intensively at the periphery of coagulation necrosis, while pyroninophilia was very slight or totally absent both in the central necrotic and in the marginal areas.

No essential changes were observable in the 48-hour specimens. The contours of trabeculae were still detectable in necrosed areas. The difference between this and the 24-hour pattern was only in that the necrosed part gave almost no staining with Feulgen's stain or with methylgreen and pyronin and that the marginal zone that stains green has slightly increased in diameter.

After 144 hours the pattern was still the same, except that homogenisation of the structure was even more marked in the necrosed part.

The histologica lfindings in both tumour and liver tissue were in complete agreement with the results of chemical tests and have confirmed the latter. On their basis it could also be explained why chemical estimations did not show a decrease of the DNA content. Although there were dissolution of cellular

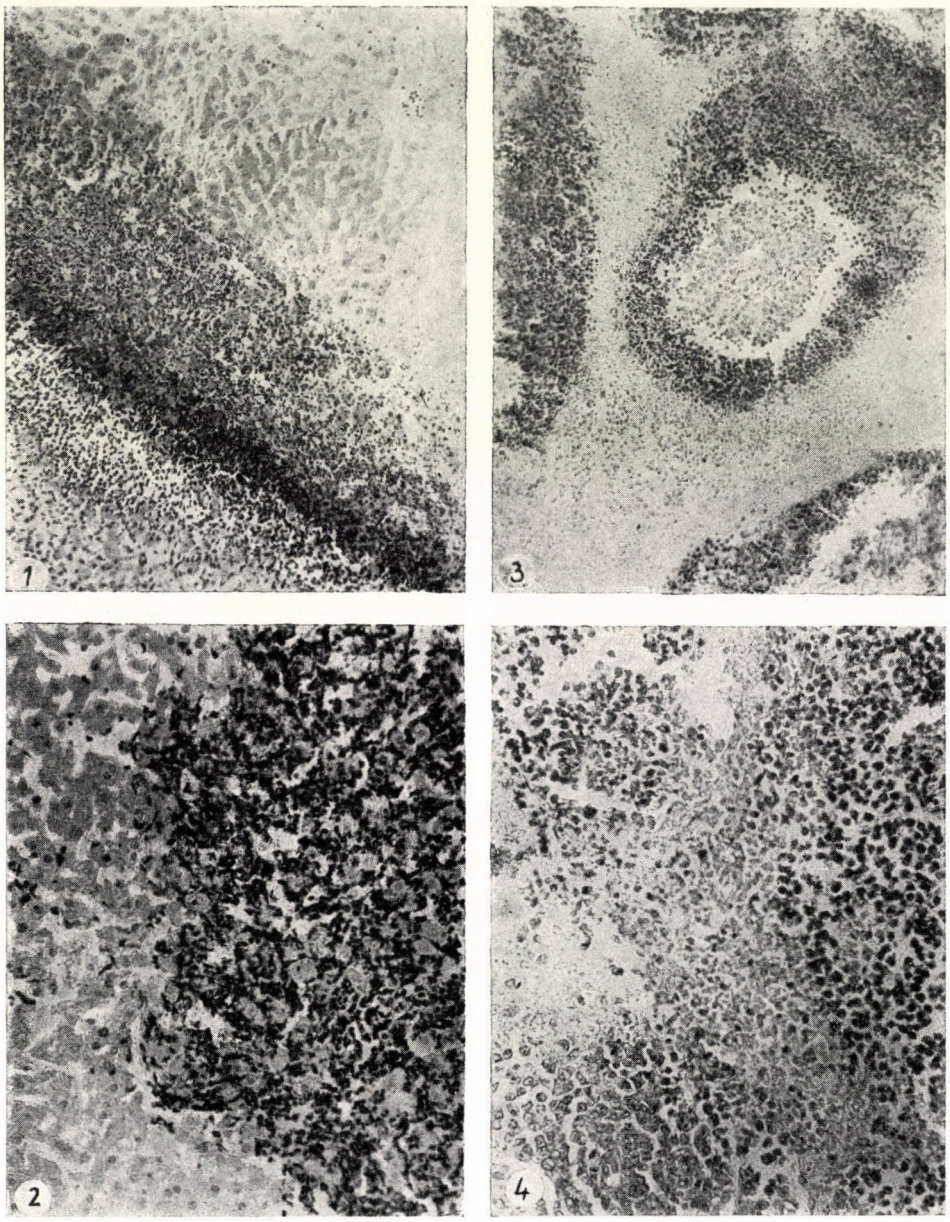

Fig. 6. 1. Necrosis of the liver. HE stain. ×80. 2. Necrosis of the liver. Methylgreen and pyronin stain. ×160. 3. Guérin tumour. Feulgen's stain. ×80. 4. Guérin tumour. Methylgreen and pyronin stain. ×160

structure and absence of nuclear staining in the necrosed zone, indicating that DNA had left that area, the same DNA had remained present in a chemically unchanged form and in the same quantity at the periphery of the necrosed areas, in the cellular detriment, pyknotic nuclei and accumulations of chromatin. By gross separation, however, the whole necrotic mass is homogenized at once, so that at quantative estimation an unchanged amount of DNA will be found.

The decrease in RNA, could be confirmed histochemically by staining with methylgreen, and pyronin, since pyroninophilia has been found to have significantly diminished in both the necrosed areas and the marginal zone of disjuncted cells.

DROCHMANNS reported similar results, although he had induced liver necrosis by ligating the portal vein. In his histochemical studies on necrosed liver tissue it was found that 24 hours after operation there was extensive intralobar necrosis, the cytoplasm did not take basic dyes, while the nuclei were still Feulgen-positive and basophilic. 24 hours after ligating the portal vein only the periphery of the lobes had remained normal, but there was a zone or well-defined outlines interposed between the marginal normal and central necrotic areas and in that zone the alterations were even more marked than in central parts. Nuclear staining was absent, but the cells of Kupffer contained more RNA than in other areas.

In view of the fact that in our experiments we had ligated all the hilar vessels leading to the hepatic lobe, it can be understood why we have found no normal zone in the lobe, nor was there a zone composed of destroyed tissue interposed between the central and the peripheral zones.

Summary

The Guérin tumour contains equal amounts of DNA in both the florid and necrosed parts. The latter contain considerably less RNA than intact parts.

The same can be stated for necrosis of the liver induced by ligation of the hepatic lobe.

Results of histochemical studies involving haematoxylin-eosin, Feulgen and methylgreen-pyronin staining, were in highly satisfactory agreement with the above findings, in both the Guérin tumour and in necrosis of the liver.

Finally, it can be stated that RNA is broken down in, and is removed from the necrosed parts of the tumour, while DNA remains detectable in a chemically unchanged form and in unchanged quantities in the cellular detriment, pyknotic nuclei and accumulations of chromatin especially at the periphery of necrosed areas.

Acknowledgements

Author expresses her thanks to DR. A. NAGY, for performing the hepatic ligations and to DR. J. SUGÁR, for his assistance in the histological studies.

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[СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ НУКЛЕИНОВЫХ ВЕЩЕСТВ ЖИВЫХ И НЕКРОТИЗИРОВАННЫХ ТКАНЕЙ

Е. ВИГ

Приведенные исследования относятся к нуклеиновым веществам омертвевших тканей в случае опухоли Герена и искусственно вызванного некроза печени.

В ходе опытов автор тщательно отделил некротизованные части от неповрежденной, сохраненной опухолевой или же печеночной ткани, а затем он определил отдельно как в некротической, так и в живой части различные нуклеиновые вещества на основании их содержания фосфора.

Обработаны были 5 опухолей, печени 36 оперированных, как и 5 контрольных крыс.

Результаты исследований следующие : содержание дезоксирибонуклеиновой кислоты как живой, так и омертвевшей части было во всех случаях одинаковым, в то время как для рибонуклеиновой кислоты были выявлены значительно более низкие величины в омертвевших, чем в живых частях.

Соответствующее исчезнувшему количеству рибонуклеиновой кислоты количество фосфора автор не обнаружил и в других фракциях омертвевшей массы.

Подобные результаты были получены также в случае некроза печени, искусственно вызванного накладыванием лигатуры на гилюсные образования, ведущие к 1—2 долям печени крыс.

В случае некроза печени автор проводил также наблюдения за течением некроза во времени. Установлено, что содержание дезоксирибонуклеиновой кислоты от 6—96 часов после операции также уменьшается, но спустя 144 часа в большинстве случаев уже вновь достигает первоначального уровня.

Содержание рибонуклеиновой кислоты уменьшалось через 6 часов после операции на 50%, а после 24 часов оно уменьшалось до 15—20% первоначальной величины, и оставалось на этом уровне даже через 240 часа после операции.

Проведенные гистохимические исследования во всех отношениях утвердили результаты химических исследований.

Автор проводил также исследования гематоксилинэозиновой и метилгрюн-пирониновой окраской, как и реакцией Фёльгена, и установил, что по результатам всех этих исследований окрашивание ядер почти совершенно исчезает из некротических областей, однако, на периферических частях этих областей, еще в некротической массе, ядерное вещество, то-есть собственная дезоксирибонуклеиновая кислота накопляется в виде обломков ядер, пикнотически сморщенных ядер и хроматиновых скоплений. Этим объясняется, что количество дезоксирибонуклеиновой кислоты осталось без изменения.

Результаты гистохимических исследований также выявляли, что рибонуклеиновая кислота исчезает из омертвевших частей опухоли или же печени.

VERGLEICHENDE ANALYSE DER NUKLEINSUBSTANZEN FLORIDER UND NEKROTISCHER GEWEBE

EDITH VIG

Die angeführten Analysen beschäftigen sich mit den Nukleinsubstanzen nekrotischer Gewebe im Falle von Guèrin-Tumoren und künstlich hervorgerufener Lebernekrose.

Im Verlauf der Untersuchungen hat Verfasserin die nekrotisierten Teile sorgfältig von dem intakten, konservierten Tumor- bezw. Lebergewebe abgesondert, sodann sowohl im nekrotischen, als auch im floriden Teil die verschiedenen Nukleinsubstanzen auf Grund ihres Phosphorgehaltes bestimmt.

Es wurden 5 Tumoren und die Leber von 36 operierten, sowie von 5 Kontroll-Ratten verarbeitet.

Die Ergebnisse waren die folgenden : der Inhalt des floriden und des nekrotischen Teiles an Desoxyribonukleinsäure (DNS) wurde in allen Fällen gleich gefunden, während die Ribonukleinsäure (RNS) im nekrotischen Teil wesentlich niedrigere Werte zeigte, als im floriden Teil.

Die der verschwundenen RNS-Menge entsprechende Menge von Phosphor wurde auch in den übrigen Fraktionen der nekrotischen Masse nicht vorgefunden.

Auch im Falle künstlicher Lebernekrose, die durch Unterbindung der zu 1—2 Läppchen der Rattenleber führenden Hylusgebilde hervorgerufen wurde, sind die Resultate ähnlich.

Im Falle von Lebernekrose wurde auch der zeitliche Ablauf der Nekrose untersucht. Es wurde festgestellt, dass sich die DNS in einem Zeitraum von 6 bis 96 Stunden nach erfolgtem Eingriff gleichfalls verringert, nach 144 Stunden aber in der Mehrzahl der Fälle wieder ihr ursprüngliches Niveau erreicht.

Die RNS sank 6 Stunden nach dem Eingriff auf 50%, verringerte sich sodann auf 15—20% des ursprünglichen Wertes und verblieb auch 24 Stunden nach dem Eingriff auf diesem Niveau.

Die histochemischen Untersuchungen bestätigten in jeder Hinsicht die auf chemischem Wege gewonnenen Resultate.

Bei den mit Hämatoxylin-Eosin-, Feulgen- und Methylgrün-Pyronin-Färbungen vorgenommenen Untersuchungen wurde festgestellt, dass die Kernfärbung bei jeder der drei Methoden aus den nekrotischen Teilen zwar fast vollständig verschwindet, die Kernsubstanz aber, d. h. im wesentlichen die DNS, sich am Randgebiet, jedoch in dem noch zur nekrotischen Masse gehörenden Teil, in Gestalt von Kerntrümmern, pyknotisch zusammengeschrumpften Kernen, sowie von Chromatinhaufen anhäuft. Damit erklärt sich auch die unveränderte Menge an DNS.

Die RNS entleert sich auch gemäss den histochemischen Untersuchungen aus dem Tumor bezw. den nekrotischen Teilen der Leber.

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