Proceedings of the Iowa Academy of Science

Volume 67 | Annual Issue Article 65

1960

Some Aspects of Quantitative Histology of the Liver of A-Jax Mice Before and During Tumor Induction with Carbon Tetrachloride

Paul A. Meglitsch
Drake University

Robert R. Mote
Drake University

Leland P. Johnson
Drake University

Some Aspects of Quantitative Histology of the Liver of A-Jax Mice Before and During Tumor Induction with Carbon Tetrachloride

PAUL A. MEGLITSCH, ROBERT R. MOTE, and LELAND P. JOHNSON[1]

Abstract. The proportion of the cell populations composed of parenchymal, littoral, and other types of cells is reported for control mice, mice repeatedly anesthetized, mice fed with olive oil repeatedly, and mice fed with carbon tetrachloride and olive oil, showing that the cellular components undergo a marked change when carbon tetrachloride is administered. The proportion of the liver volume occupied by parenchymal cells is found to be relatively stable throughout the period of tumor induction. The nuclear-cytoplasmic ratio of parenchymal cells is found to fall with the aging of control mice and those fed olive oil or anesthetized during the period of the experiment. The nuclear-cytoplasmic ratio is found to fall to a minimal point during the first two weeks of carbon tetrachloride feeding, thereafter gradually rising to normal values for mice of the same age, and eventually rising, after 24 feedings, to a value exceding that of normal mice of the same age. Between 24 and 30 feedings the nuclear-cytoplasmic ratio undergoes a decrease. The volumes of parenchymal cell nuclei are found to vary with the position in a lobule, the maximal nuclear volumes being found in a region about 100μ to 200μ from the central vein in lobules of average size.

It has long been known that the ingestion of carbon tetrachloride results in liver damage and is followed by replacement of liver cells. The mechanical removal of liver tissue also evokes the replacement of liver tissue by hyperplasia of intact lobes. Early work in this field is reviewed by Fishback (1929). Repetitive damage with carbon tetrachloride was found to lead to the induction of hepatomas in a number of animals. Dalton and Edwards (1942) characterized the normal and tumorous tissues so induced in mouse liver, insofar as histological structure is concerned. Since this time a number of investigators have studied liver regeneration and the induction of hepatomas with carbon tetrachloride using rodent material. The majority of the work has been centered about the use of rats and the A-Jax strain of mice. A variety of approaches has been used. Biochemical, histochemical, and more detailed histological studies have been carried out.

Chemical characterization of tumorous and normal liver tissues and comparison of enzymatic and other properties of mitochondria from homogenates of tumorous and normal tissues have been under-

[1]Department of Biology, Drake University, Des Moines, Iowa.

taken by a number of investigators. Greenstein (1947) reviewed much of the earlier work in this field. Although the use of homogenates has provided a great deal of important information, some serious problems have arisen in interpreting the results. Most of these problems center about the determination of an adequate reference base. Early work was usually reported in units related to the volume or weight of the liver tissue used in the homogenates. Tsuboi, Stowell, and Lee (1951), however, have shown that regenerating liver tissue undergoes changes in water content as well as in specific gravity. Thus two homogenates which have identical enzymatic activity or chemical content on a per unit of weight basis may differ significantly if differences in specific gravity are taken into account. Moreover, the actual size of the liver cells does not remain constant during tumor induction or liver regeneration, so that differences observed in chemical parameters on a per unit weight basis may not be correlated with changes in the content of individual cells.

It seemed reasonable, therefore, that biochemical results should be referred to a cellular base. Allard *et al.* (1952), for example, based their data on a per nucleus figure, nuclear numbers being determined by direct count of samples of the nuclear fraction of the homogenate. Others have suggested that the data be reported per unit of DNA. The work of Beams and King (1942), and others, indicated that the use of numbers of nuclei or units of DNA as a reference base is by no means safe. There are many binucleate cells in liver tissue, and the percentage of binucleate cells is not stable but changes with age or under different experimental conditions. In addition, ploidy differences among nuclei in the liver tissue introduce another source of error. Above all, as Daoust (1958) pointed out, a direct count of nuclei in homogenates can at best tell only approximately how many cells are present; but it does not permit distinction between the cells of various kinds. It has already been shown that the relative abundance of some of the types of cells undergoes significant changes at different times during liver regeneration, and this introduces still another potential source of error in interpreting the significance of the results of the study of homogenates. The solution, as Daoust, among others, has pointed out, is to characterize more carefully the average liver cell, on a quantitative basis. This is a preliminary report of work undertaken to describe more adequately the composition of the average cell in the liver of A-Jax mice during and after tumor induction with carbon tetrachloride.

The authors wish to express their appreciation of the financial support given for this work by the American Cancer Society, Grant E-199.

Materials and Methods

Female A-Jax mice obtained from the Jackson Memorial Laboratory were used. At an age of 6 weeks (plus or minus ½ week) the mice were placed on a regimen. One lot were kept as controls without treatment. Another lot were anesthetized 3 times per week with ether, but in other respects treated like control mice. A third lot were anesthetized and fed 0.1 cc of olive oil 3 times weekly, and in other respects handled like the control mice. A fourth lot were given 0.1 cc of a 40 percent solution of carbon tetrachloride in olive oil following anesthesia 3 times weekly, and in other respects handled like the controls. Mice were sacrificed at 2 week intervals from the carbon tetrachloride series. Control mice were sacrificed after 6, 8, and 10 weeks. Mice repeatedly anesthetized were sacrificed after 8 weeks, and mice fed olive oil were sacrificed after 6, 8 and 10 weeks.

Animals were killed by a blow on the head administered 24 hours after the last anesthesia or feeding. Samples of tissue were removed from the peripheral and basal portions of the larger liver lobes and preserved in a number of fixing solutions (Zenker's, Zenker's without acetic, Helly's, Hoyer's, Regaud's and Maxinow's fluids). Following standard washing techniques, the samples were imbedded in paraffin for sectioning. Sections were cut at 3 μ and 6 μ or 8 μ, and stained with Delafield's haematoxylin and eosin, Heidenhain's iron haematoxylin, Mallory's triple, and Bensley-Cowdry's fuchsin-methyl green.

Chalkley's (1943) point ratio method was used to determine the relative volume of different components of the liver. In this method the slide is moved and focused at random, and the position of a point is recorded as being in the nucleus, or cytoplasm, of a parenchymal cell, in a littoral cell, a blood cell, vascular space, bile duct cell or lumen, or a connective tissue cell. The ratio of hits in the various liver components to the total number of points recorded approaches the ratio of volumes of the tissue components.

With the aid of an ocular reticule, the number of nuclei or nuclear fragments occurring in a definite area of a section was also determined. The nuclei were categorized as belonging to parenchymal cells, littoral cells, blood cells, bile duct cells, or connective tissue cells. Sections cut at two prescribed thicknesses were routinely used for this study to permit more accurate correction for nuclear fragments. Data gathered in this manner may be used to estimate the total number of nuclei, and, presumably, the total number of cells of various types in a known volume of fixed liver tissue.

A third series of studies was used for determining the average value of parenchymal cell nuclei in various tissue samples, and

through the volumes, the relative frequency of nuclei falling in various ploidy categories. Camera lucida tracings were used for these measurements. The tracings were measured to the nearest millimeter in the longest and shortest diameters. It was assumed that the nuclei were oblate ellipsoids, so all volume determinations were based on nuclei with unequal diameters. Some random samples were taken, whereas in other cases a band of tissue extending from the central vein to the paraportal region was studied. In the latter case, the band was divided into zones 50μ broad, and the average nuclear volume in each portion was determined, thus giving a picture of any diversity of nuclear volumes associated with position in the lobule.

Cell Populations in the Liver

Two samples of liver having the same weight or volume may vary considerably with respect to the actual numbers and proportions of cells present. Daoust (1958), for example, reported that in rat liver only 60.6 percent of the nuclei present were from parenchymal cells; 33.4 percent were from littoral and 6 percent from other types of cells (bile duct, connective tissue, blood vessel walls). It is evident that a significant number of cells other than parenchymal are represented in a homogenate. Daoust also pointed out that in his material the average liver cell had a pliody of 2.9 n, which agreed quite well with the approximately 3.0 to 2.0 ratio of DNA per cellular unit in liver as compared with diploid organs.

Inasmuch as all of the data concerning nuclear sizes are not yet available, corrective factors to modify the number of nuclear fragments into number of nuclei actually present have not been used.

Table 1
Number of Nuclear Fragments, Expressed As Percentages of the Total Number of Fragments Counted

Material	Parenchymal Cell	Littoral Cell	Other Cell Types
Controls (6 weeks)	64.5	30.0	5.8
Controls (14 weeks)	62.0	30.1	7.9
Controls (16 weeks)	64.7	30.0	5.7
Anesthetized 24 times (14 weeks old)	63.6	31.7	4.7
Fed olive oil 24 times (14 weeks old)	66.0	30.1	3.9
Fed olive oil 30 times (16 weeks old)	63.8	32.0	4.2
Fed CCl_4 24 times (14 weeks old)	46.7	44.3	9.0
Fed CCl_4	40.4	43.5	16.5

Table 1 summarizes the raw data which have been obtained, and as the corrective formulae do not greatly change the figures, some comparisons may be made. It is evident that control samples are similar to those studied by Daoust in the rat. Parenchymal cells make up 65 percent or a little less of the total number of nuclei and fragments, whereas littoral cell nuclei make up about 30 percent of the total. The remaining cells contribute from 5.7 percent to 7.9 percent. There is no evidence that the amount of aging associated with the experiment has any appreciable effect on the proportion of cell types. Ether anesthesia and olive oil feedings appear, also, to have no effect on the relative abundance of cell types. It should be noted, however, that the application of corrective formulae which take differences in nuclear size into account may tend to magnify some of the slight differences which occur in the raw data. Feeding carbon tetrachloride changes the proportion of cell types significantly. The number of parenchymal nuclei is reduced, with compensatory increases in the number of littoral and other cell types. It has been found that the period centering about the 18th through the 30th feeding is a critical period during which hepatomas generally appear. It is interesting to note that this corresponds with the period when cells other than littoral and parenchymal are increasing very rapidly.

Although a complete analysis has not yet been undertaken, preliminary consideration of the data shows little reason to suspect that dissimilar cell populations are found in different regions of a liver lobe. The distribution of cells is, of course, related to lobular structure, but in random samples of control tissues from all parts of lobules and regions between lobules as well, peripheral and basal portions of a lobe appear to be very similar. In mice fed with carbon tetrachloride, however, there is some evidence of difference between peripheral and basal regions. In mice fed carbon tetrachloride 24 times, 9.3 percent of the nuclei are from bile duct cells in peripheral samples, but only 1.4 percent of the nuclei are from bile duct cells in basal samples. After 30 feedings the proportions are 10.5 percent and 5.3 percent, respectively. These differences appear to be significant and can be seen in individual animals as well as in averages of groups. Tissues from the basal portions of the lobe tend to have a larger percentage of connective tissue nuclei than do tissues from the peripheral portions. This difference is less consistent and less marked than in the case of the bile duct cells.

There is a considerable variation among small samples. This is to be expected, as a small sample may pass through a portal region or miss a portal region, with corresponding changes in cell frequencies. As samples accumulate, however, it becomes evident that the cell population in any given mouse liver tends to be reasonably uniform throughout, as does the proportion of cell types in different

mice that have been kept under the same conditions. The figures quoted are based on samples of over 2,000 nuclei from each mouse used, about half from the peripheral region and half from the basal region of a lobe. With samples of this size good agreement is generally found among different animals which have been treated similarly.

It appears evident that there is sufficient change during the period of aging to account for significant changes in enzyme activity or quantities of chemical substances seen in homogenates from normal mice, or from mice which have been anesthetized or fed olive oil. However, comparison of homogenates made from the livers of mice fed carbon tetrachloride with homogenates made from normal mouse livers should not be made without due reference to the changing cell populations. The proportions of cell types change to such an extent that significant differences in enzyme activity or in the quantity of a substance may result from changes in relative cell frequencies rather than from changes in the composition of the cells individually.

Table 2

The Percentage of Liver Volume Made up of Parenchymal Cells, and the Percentage of Parenchymal Cell Volume Made Up of Nuclear Material, in Livers of Mice Under Differing Conditions

Material	Parenchymal Cells	Nuclear Material
Control samples (12 weeks old)	88.8	9.4
Control samples (14 weeks old)	88.4	8.4
Control samples (16 weeks old)	90.5	8.8
Anesthetized once (6 weeks old)	83.9	10.6
Anesthetized 24 times (14 weeks old)	90.6	8.0
Fed olive oil once (6 weeks old)	89.9	10.1
Fed olive oil 18 times (12 weeks old)	90.2	10.0
Fed olive oil 24 times (14 weeks old)	87.5	10.2
Fed olive oil 30 times (16 weeks old)	89.7	8.4
Fed carbon tetrachloride once (6 weeks old)	90.0	10.3
Fed carbon tetrachloride 6 times (6 weeks old)	84.0	6.8
Fed carbon tetrachloride 12 times (10 weeks old)	87.5	8.2
Fed CCl_4 18 times (12 weeks old)	83.5	9.1
Fed CCl_4 24 times (14 weeks old)	87.0	10.1
Fed CCl_4 30 times (16 weeks old)	89.1	8.7

The Quantity of Parenchymal Cell Substance in the Liver

Determination of the percentage of the liver volume which is composed of parenchymal cell material by the Chalkley point ratio method yields data indicating that this value is relatively stable. Individual samples vary quite markedly, as Wilson *et al.* (1953) have shown. This appears to be because of differences in the relative size of the blood vessels in the portions of the liver examined, differences in the blood content of the sinusoids, and other less important factors beyond the scope of this discussion.

As shown in Table 2, the data indicate that up to the time of the critical period of tumor induction, no significant changes in the relative volume of the parenchymal substance is seen. It has been reported previously that the number of parenchymal cell nuclei is significantly reduced in the period between 24 and 30 feedings. This indicates that the individual parenchymal cells must be increasing in size during this period. There is evidence that the nuclei, considered as individual entities, are also increasing in size at this time.

The Nuclear-cytoplasmic Ratio in Parenchymal Cells

The data concerning the percentage of the parenchymal cell volume occupied by the nuclei are summarized in Table 2 and Figure 1.

Figure 1. The percentage of parenchymal cell volume composed of nuclear material. Unshaded bars represent data from control animals; cross-ruled bars, animals anesthetized with ether; diagonally ruled bars, animals fed olive oil; black bars, animals fed carbon tetrachloride. All but the control animals were treated three times weekly. The relative nuclear volume tends to fall during the later part of the experimental period in all animals except those fed carbon tetrachloride. In these there is an early, sharp reduction in the relative volume of the parenchymal cells, followed by a gradual rise up to the 24th feeding with carbon tetrachloride.

There is definite evidence that the nuclear-cytoplasmic ratio changes with the age of the mouse during the period of the experiment. Mice 6 weeks old tend to have approximately 10 percent of the parenchymal cell volume composed of nucleus. As the mouse ages, this value tends to fall. Mice from 14 to 16 weeks old tend to have a nuclear-cytoplasmic ratio of about 8.5 percent. There is little evidence to indicate that ether anesthesia or feeding with olive oil materially changes this picture, although it is not impossible that feeding with olive oil tends to reduce somewhat the speed with which the relative nuclear volume falls.

The administration of carbon tetrachloride repeatedly results in a definite modification of the normal changes in nuclear-cystoplasmic ratio. Twenty-four hours after the initial feeding with carbon tetrachloride little change in nuclear volume has occurred. After six feedings, however, the relative volume of the parenchymal nucleus has been materially reduced. The relative volume of the parenchymal nucleus rises with each fortnightly sample, reaching a maximum of 10.1 percent after 24 feedings when the mouse is 14 weeks old. After 30 feedings the nuclear-cytoplasmic ratio appears to fall once again. Although the data are consistent on this point, the quantity of data now available is just short of producing a .05 probability of difference. It may be significant that the period from 18 to 20 feedings is a critical period during which tumors appear and is also a time when the cell population is undergoing a marked shift. It is not improbable, therefore, that there is a real shift in the direction of change of the nuclear-cytoplasmic ratio at this time.

Nuclear Volumes

Data are incomplete with respect to nuclear volumes. It is evident, however, from the data so far obtained that the parenchymal cells in a lobule are not randomly distributed insofar as nuclear volume is concerned. If the distance between central vein and portal region of a lobule is divided in 50 μ zones, the average volume of nuclei in the various zones shows a regular change in all samples so far analyzed. The nuclei are smallest in the paraportal region. In each consecutive zone more distant from the portal the average nuclear volume rises (Figure 2), reaching a maximal value in the zone between 100 μ and 150 μ or 150 μ to 200 μ distant from the central vein, the position apparently depending on the size of the lobule. From the zone of maximal nuclear volume, the nuclei decrease relatively rapidly in size, reaching a small size in the zone adjacent to the central vein. The nuclei in this zone have a noticeably larger volume than those adjacent to the portal vein, however.

In any given mouse the average nuclei volume of the parenchymal cells in a lobule remains relatively constant. Two lobules of approxi-

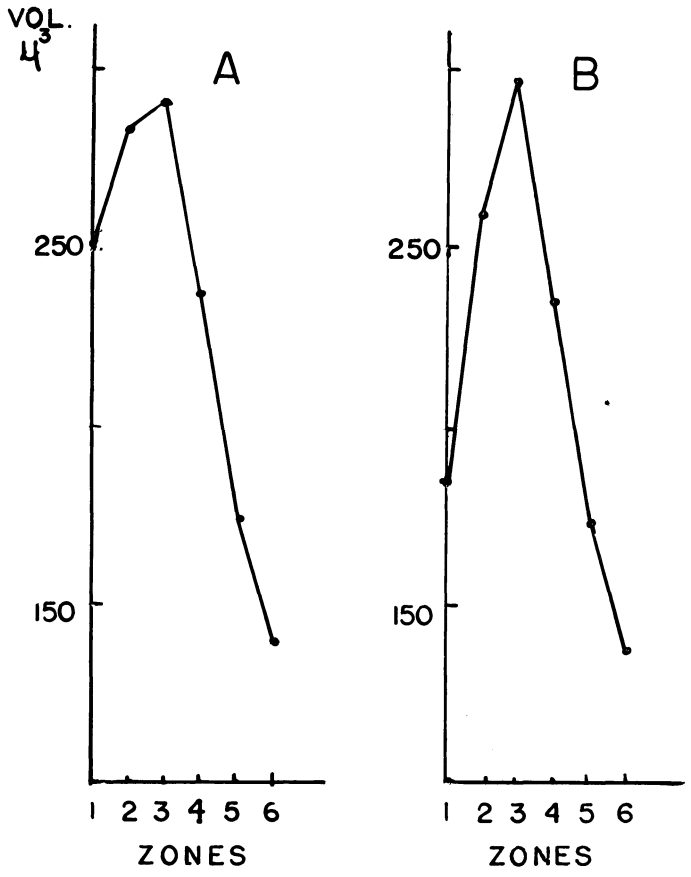

Figure 2. The mean volume of parenchymal cell nuclei as a function of position in a lobule. All nuclei found in zones 50μ across are averaged together to obtain the main nuclear volume of a zone. Zone 1 is adjacent to a central vein, and each succeeding zone is peripheral to the preceding zone. The sixth zone in each of the two lobules whose profiles are shown is adjacent to the portal vein. *A* shows the profile in a peripheral portion of a liver lobe; *B* shows the profile in a basal portion of the same liver lobe in the same animal.

mately the same size, one from the periphery and the other from a basal region of a lobe, have nearly identical nuclear volumes in each region from the portal to the region of maximal volume. The zones adjacent to the central vein appear to be somewhat more variable. These zones also tend to have somewhat more diversity in terms of ploidy groups.

Since the work of Beams and King (1942) it has been generally understood that the parenchymal cells of the liver vary with respect to their chromosomal content. The volumes of the nuclei vary with their ploidy category, the tetraploid nuclei having approximately double the volume of the diploid nuclei, etc. Observations so far have

confirmed this principle. It is clear that the frequency of diploid nuclei is highest in the portal region and reaches a minimum in the region of maximal nuclear volume. In several lobules, not a single diploid nucleus was found in this zone. There is some evidence that the average size of the diploid nuclei is also a variable, with the smallest diploid nuclei occurring adjacent to the portal vein.

Discussion

It is evident that the histological content of the liver is variable. Some factors are modified with age, whereas others remain relatively stable during the period of the life cycle that has been covered. Other factors are modified by experimental conditions. The histological differences, as they are quantified, can serve an important role in making a more adequate interpretation of biochemical work. Every quantitative method that has thus far been applied to the study of the liver has revealed differences, of one kind or another, of sufficient magnitude to be a factor capable of explaining some kinds of differences which homogenates show.

The regular profile of changing nuclear volumes in parenchymal cells, associated with their position in the lobule, is interesting in its potential relationship to differences in the activities of the cells. Noel (1923) conceived of the liver cells as falling into three zones, a zone of repose, an intermediate zone, and a zone of activity. The largest nuclei are found in cells which would undoubtedly fall in Noel's intermediate zone. Schepers (1960) described the portal region as the site of liver cell formation, and the intermediate region as the zone of most intense liver cell activity. If this view proves to be correct, the smallest nuclear volumes occur in the region of liver cell formation, whereas the largest ones occur in the area of maximal activity. Declining nuclear size, in this instance, would occur in senile cells as they approach the central vein.

Literature Cited

Allard, C., Mathieu, R., de Lamirande, G. and Cantero, A. 1952. Mitochondrial population in mammalian cells. Cancer Res. 12: 407-412.

Beams, H. W. and King, R. L. 1942. Origin of binucleate and large mononucleate cells in liver of rat. Anat. Rec. 83: 281.

Chalkley, H. W. 1943. Method for the quantitative morphologic analysis of tissue. J. Nat. Cancer Inst. 4: 47-53.

Dalton, A. J., and Edwards, J. E. 1942. Mitochrondria and Golgi apparatus of induced and spontaneous hepatomas in the mouse. J. Nat. Cancer Inst. 2: 565-575.

Daoust, R. 1958. The cell population of liver tissue and the cytological reference bases. Liver function. Ralph W. Brauer, ed. American Institute of Biological Sciences, Washington, D. C.

Fishback, F. C. 1929. A morphologic study of regeneration of the liver after partial removal. Arch. Path. 7: 955-77.

Greenstein, J. P. 1947. Biochemistry of cancer. New York, N. Y. Academic Press Inc.

Noel. R. 1923. Sur la cellule hepatiques des mammifères. Arch. d'Anat. Microscopique 19: 1-158.

Schepers, G. W. H. 1960. The fate of liver cells. Federation Proc. 19 (1): 185.

Tsuboi, K. K., Stowell, R. E., and Lee, C. W. 1951. Chemical alterations induced in mouse liver following a single feeding of carbon tetrachloride. Cancer Res. 11: 87-93.

Wilson, M. E., Stowell, R. E., Yokoyama, H. O. and Tsuboi, K. K. 1953. Cytological changes in regenerating mouse liver. Cancer Res. 13: 86-92.