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Focusing experimentally on polyploidy in physiology and pathology of mammals

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Dedicated to the memory of Vincenzo Chiarugi (1939-2002), professor of molecular biology, who worked all his life in the Department of General Pathology and Oncology of the University of Firenze, formerly Institute of General Pathology[§].

Abstract — This paper, aimed at focusing on polyploidy in mammalian cells with attention to pathology, consists of two parts. The experimental part shows the results of a retrospective morphometric analysis carried out on histological specimens of toxic fatty livers saved together with the inherent records of year 1959. Some polyploid anaphases detected merely by chance and suitable to be quantitated have been analysed by computer-assisted microscopy. The results of this analysis prove that genome amplification may occur even in 16n hepatocytes infiltrated by lipid droplets and, presumably, heavily swollen. This information is relevant to re-interpret correctly pathophysiology of toxic fatty liver. In addition, this paper traces the outline of present knowledge on genome amplification in normal and pathological mammalian cell types. Morphometric data and recent progress of molecular cell biology and genetics are indicative of the importance of ploidy values in connection (i) with differentiation, growth and aging of the normal organism, (ii) with the so-called final differentiation of some cell types, and (iii) with some processes closely linked together, namely, inflammation and repair. Evidence suggests that in the life history of the organism mammal polyploidy is controlled, in the various organs, tissues and cell lines, and/or at different times, by different genetic programs.

Key words: polyploidy, mammals, multinucleated cells, polyploidy in pathology.

INTRODUCTION

At the beginning of the era of molecular biology, it was understood that gene duplication, that is, polyploidization, plays a basic role in evolution (OHNO 1970). Today the importance of polyploidy, from unicellular organisms to mammals (ADAMS *et al.* 2003; MCLYSAGH *et al.* 2002; OSBORN *et al.* 2003; and references therein) in triggering epigenetic changes (SCHEID *et al.* 1996; GALITSKY *et al.* 1999; KELLOGG 2003; NAGATA *et al.* 2005) and in breaking down taxonomic barriers (MABLE 2003) is widely accepted, and the interest for the various faces of this issue is proved by the number of published papers. It is worth recalling here that the importance of polyploidy in pathophysiology of mammalian cells and organs has been recognized since at least four decades, mainly in connection with both normal growth and hypertrophy of the heart (SANDRITTER and SCOMAZZONI 1964) and with normal growth and regeneration of the liver (EPSTEIN 1967; NADAL and ZAIDELA 1966). Other facts equally important, also known since much time, are that viruses, drugs, and changes in ionic strength may induce polyploidization of cultured cells (historical survey in: EPHRUSSI 1970) and that monoclonal antibodies are produced by heterokaryons obtained by cell fusion, also called hybridomas. An exhaustive review on polyploidy in normal cells of mammals, published in this Journal many years ago (D'AMATO 1989) is still cited in the literature (ANATSKAYA and VINOGRADOV 2004). D'AMATO's paper dealt with normal tissues and reported in detail on the different types of cell polyploidy, an issue updated by ZIMMET and RAVID (2000). Polyploidization was given much attention by a valuable textbook of general pathology (MAJNO and JORIS 1996), and has been updated in a recent paper on Purkinje neurons (DEL MONTE 2006). Cur-

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rently, studies going on in a number of laboratories, mainly with methods and techniques of molecular biology and molecular genetics, are concerned more with biomedical sciences than with evolution. However, at variance with trends of modern biomedicine, several textbooks of cellular and molecular biology still give scarce, if any, attention to this issue and the word "polyploidy" is rarely found in their glossaries and subject indexes.

Insightful principles of genetics governing gene expression may be of help in re-interpreting cellular pathology. A few polyploid anaphases detected by chance in this laboratory and deserving further attention offered the opportunity to focus on polyploidy in mammals. These mitotic figures of polyploid hepatocytes from rats treated with steatogenic poisons are, in fact, clear-cut examples of scaling of DNA linked with scaling of cell size during postnatal maturation of mammalian hepatocytes up to a polyploid phenotype, linked, perhaps, with terminal differentiation (for terminal differentiation of hepatocytes, see: GUPTA 2000). However, while this study was in progress, a paper claiming that Purkinje neurons are tetraploid (LAPHAM 1968) diverted for some time my attention on the puzzle of polyploidy of the terminally differentiated neural cells (DEL MONTE 2006).

EXPERIMENTAL

Biological Material - Sacrifice of animals was not required. In fact, anaphases of large, apparently polyploid hepatocytes were found while re-examining, in connection with a study on cellular swelling (DEL MONTE 2005), some HE-stained paraffin sections dating back to old experiments described in a paper on the effects of steatogenic poisons on rat liver (DEL MONTE and FONNESU 1959). The polyploid hepatocytes shown in Figure 1 B and 1 C are located in histological sections from livers of two adult 2-3 month-old female rats which had been treated respectively: for six days with a subcutaneous injection of carbon tetrachloride (0.2 ml/100 g body wt./day of a 20% solution of the poison in olive oil), and for five days with a subcutaneous injection of white phosphorus (0.05 ml/100 g body wt./day of a 0.5% solution of white phosphorus in olive oil). The replicating diploid hepatocytes were selected in two histological sections from an old set of liver specimens from 14 day-old suckling rats (CAPACCIOLI *et al.* 1977), at which time the liver contains "only mononuclear diploid cells" (GUPTA 2000). Resting cells (endothelial cells, lymphocytes) were selected as 2n reference standards in the same microscopic fields of the 2n+2n replicating hepatocytes.

Histology - Most mitotic figures were not suitable to be examined, because of their unfavorable orientation within the thin (<10 μm) histologic sections. However, some anaphases with the two sets of daughter chromosomes falling in the plane of the slice could be found (Figure 1: A, B and C). Likely, the two larger hepatocytes (B and C) are flattened in shape by the mechanical pressure of the surrounding cells. Indirect proof of their flat shapes is that their round or cubic volumes calculated from the average diameters in the figure would be disproportionately higher than the volumes of the diploid dividing hepatocytes (exemplified by A). The thickness of the spindle, very similar in the three anaphases, suggests that the three cells examined are in the same period of the anaphase transition.

Computer-assisted microscopy and estimation of ploidy level in HE-stained paraffin sections - The pictures of anaphases A, B, C (Figure 1) were taken the same day under the same light conditions with an Ikegami television chamber connected with a Zeiss microscope mounting the objective 25x. The reticulum of a Thoma Zeiss chamber provided the 50 μm reference. The areas with the images of the two groups of chromosomes were cut from enlarged figures of A, B and C, and weighed with an electrobalance (0.1 mg tolerance). An independent operator measured the area of these images with a computer equipped with the program Image Pro Plus, version 4.5.1. and carried out the measurements of integrated optical density (IOD) on replicating and quiescent diploid cells as shown in Figure 1 and specified below.

Results - Figure 1 shows three anaphases (A, B, C) and their presumed ploidy values. On the left of A is schematized a quiescent (Q) diploid cell (2n). The anaphase A pertains to a diploid replicating hepatocyte (2n + 2n). Data for chromosomal areas of A, B and C, obtained either manually or by image analysis and normalized by equalizing the C values, are reported on an arbitrary scale in the inset down on the right. The graphs obtained by the two methods are nearly superimposed, the chromosomal areas for B and C being about four and eight times greater than for A. It has been assumed that approximate differences in chromosomal areas (ordinate) correspond to exact differ-

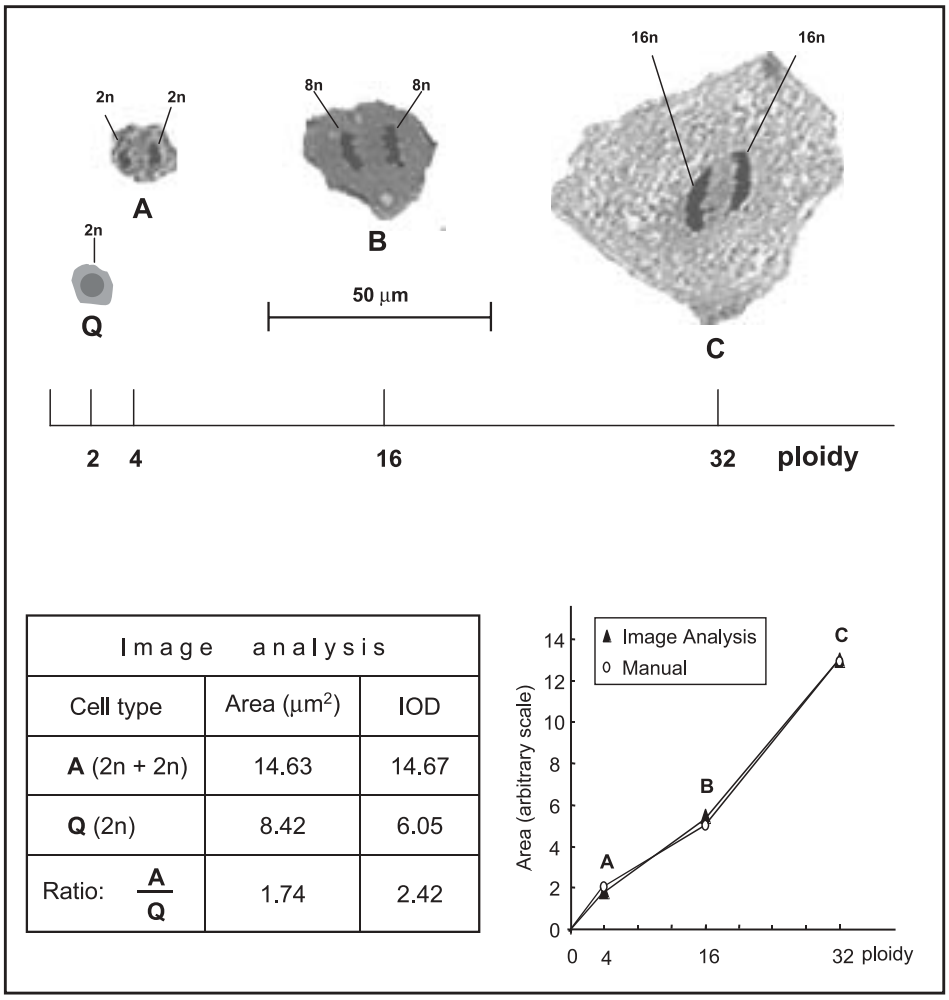

Image analysis		
Cell type	Area (μm^2)	IOD
A (2n + 2n)	14.63	14.67
Q (2n)	8.42	6.05
Ratio: $\frac{A}{Q}$	1.74	2.42

Fig. 1 — Analysis of the anaphases of diploid and polyploid hepatocytes of the rat. For details, see text.

ences in ploidy (abscissa). Slight divergences from an exact straight line in the graph should be attributed to the limits of the methods and, probably, to some chromosomal DNA cut out from the histological slice. In Figure 1 the inset down on the left summarizes in a table the results of a direct comparison of the area (in μm^2) and of the integrated optical density (IOD), of diploid dividing hepatocytes (A) with diploid quiescent cells (Q) present in the same image as required to calibrate the system for IOD (Thunnissen *et al.* 1997). In the two independent measurements averaged in the table, IOD values for the reference diploid cells (means ± Standard Deviations of four) were: 5.02 ± 0.62 and 7.08 ± 0.26. Average A/Q ratios not too distant from 2, found for both areas and IOD in these cells, allow to infer that also the chromosomal areas in B and C represent fairly well the relative amounts of DNA.

POLYPLOIDY IN MAMMALS

Polyploid is a cell containing more than two sets of homologus chromosomes whereas polytene chromosome is a chromosome in which DNA has undergone repeated replications without separation into new chromosomes (Alberts *et al.* 2002). Previously it has been stated that "cytophotometry established a fundamental similarity between polyploidy and polyteny, their common feature being multiple doubling of DNA in the cell" (Brodsky and Uryvaeva 1977) and that "the basic structure of a polytene chromosome must be similar to that of a normal chromosome" (Alberts *et al.* 1982). Anyhow, under the term "polyploidy" are combined morphologically different phenomena, to be distinguished when discussing specific cases. Likewise, polyploid mononucleated cells should be distinguished from

multinucleated cells, sometimes called polykaryons (HEYMANN *et al*. 1998). Some examples of mononucleated polyploid mammalian cells are reported in Table 1, together with examples of multinucleated cells: The latter are specialized cells (megakaryocytes, osteoclasts, etc.) normally present in certain mammalian tissues, or other kinds of giant cells accumulating under pathological conditions such as chronic inflammations and neoplasias (giant cells are listed, for instance, by SANDRITTER and THOMAS 1979). However, knowledge on this topic is increasing rapidly, and it is possible that some data in Table 1 may require revision in next future. For instance, the debated case of Purkinje neurons, claimed to be tetraploid (LAPHAM 1968), is particularly impressive. Data available, reviewed in detail elsewhere (DEL MONTE 2006**),** led (i) to note that Purkinje neurons can tetraploidize by fusing with stem cells from bone marrow (WEIMANN *et al*. 2003; MEZEY *et al*. 2003) and (ii) to suggest that their number might increase with aging, (according with a hypothesized general behaviour of somatic mammalian cells), or because a persistent stimulation of some specific function(s) leads, with time, to cell hypertrophy and polyploidization. These suggestions are consistent with present knowledge on polyploidy, a phenomenon recognized as a common event whose frequency in several tissues and organs of mammals tends to increase in relation with age. Polyploidization of somatic mammalian cells during adulthood, first documented for hepatocytes much time ago (references in: EPSTEIN 1967; GUPTA 2000), has been recently confirmed in cultured endothelial cells undergoing replicative senescence (WAGNER *et al*. 2001), and, *in vivo*, in aortic vascular smooth muscle cells (JONES and RAVID 2004), in cardiac myocytes and many other cell types (references in NAGATA *et al*. 2005). Briefly, this is the right time to point out that polyploidy influences gene expression, and may arise in different circumstances summed up in a few lines: (i) the position of the cells in one or other of the complex structures forming anatomic and functional compartments within specialized organs (such as: the lobuli or the acini in the liver;

Table 1 — Ploidy levels in different mammalian cell types.

Cell type	ploidy level	number of nuclei	Conditions		References
			physiological	experimental or pathological	
Human myocardial syncytium	2n to 16n	?	Polyploidy parallels normal growth of heart	Heart hypertrophy	SANDRITTER and SCOMAZZONI, 1964
Hepatocytes	from 2n to 16(32)n	1-4	Polyploidy parallels the enlargement of the liver with growth of the body	– liver compensatory growth following partial hepatectomy; – necrosis or apoptosis	BRODSKY and URYVAEVA, 1977 FAUSTO and WEBBER, 1994 MAJNO and JORIS, 1996
Rat (age: 2-3 months)	16n (x2?)			– cloudy swelling and steatosis induced by poisoning with white phosphrous or CCl_4	DEL MONTE, 2007 [present paper]
Neurons (mouse, spinal cord)	2n	1	Adult		SWIFT, 1953
Oligodendrocytes (human cerebellum)	2n	1	Described at any age		LAPHAM, 1968
Purkinje neurons (human cerebellum)	4n	1	Described at any age		LAPHAM, 1968
Mouse	2n	1		From BMS	PRILLER *et al*, 2001
Mouse and human	2n+2n	1-2	Fusion with BMS	Fusion with BMS	WEIMANN *et al*, 2003 MEZEY *et al*, 2003
Renal tubule cells (human)	2n				LAPHAM, 1968
Megakaryocytes	4n to 128n (average 16n)	4 to dozens	Megakaryocytes with a minimum of 4 nuclei are considered mature	Platelet diseases	PENINGTON *et al*., 1976 ZIMMET and RAVID. 2000 SUN *et al*, 2006
Osteoclasts	n.d.	some dozens	Normal bone turnover	Bone resorption	TINTUS *et al*., 2002
Macrophages	n.d.	1 to dozens	1 or few nuclei	multinucleated cells of granulomas (tubercolosis; sarcoidosis; foreign bodies; etc.)	HEIMANN *et al*., 1997 HEIMANN *et al*., 1998
Endothelial cells (HUVEC)	2n to 4n	n.d.	Aging *in vitro* (replicative senescence) is accompanied by polyploidy		WAGNER *et al*., 2001
Lymphoid cells	n.d.	normally 1 pathol. 1 to 3	Normal thymus and lymph nodes; blood and lympha	Multinucleated Reed-Sternberg cells of Hodgkin's lymphoma	KUPPERS, 2002 KUPPERS and HANSMANN, 2005

n.d. = not described; BMS = Bone marrow stem cells.

the microcomplexes in the cerebellum); (ii) the microenvironment; (iii) the repair of cells damaged by stressors (hypoxia, chemical poisons, etc.); (iv) a combination of pathological agents with some other factors, first of all, an increased functional demand lasting for a long time.

DISCUSSION

Based on the histological pictures, Figure 1 suggests that the anaphases A, B and C originating from the 2n, 8n and 16 n hepatocytes will give origin, respectively, to two 2n, two 8n and two 16 n new hepatocytes. However, the formation of binucleated cells (NAGATA *et al.* 2005) [which, in turn, by nuclear fusion (D'AMATO 1989; GUPTA 2000) might produce 4n, 16n and 32 n mononucleated hepatocytes] cannot be ruled out. Most importantly, however, the anaphases B and C document that the damage caused by the two steatogenic poisons was not great enough to preclude DNA replication by these already hyperploid hepatocytes. Furthermore, these data with two-three month old (rather young) rats are not at variance with the idea that the increased ploidy causes a decrease of the attitude to cell replication since this limitation might occur in aging and perhaps when the cells reach the so-called "final differentiation", a "physiological"concept, however, that in author's opinion can be hardly applied with the same criteria to any cell type (for instance, hepatocytes and neural cells). Since this paper was aimed at focusing on polyploidy in mammalian cells with attention to pathology, it is worth mentioning here the aberrant tetraploidization of hippocampal neurons in Alzheimer's disease leading to cell death (RAINA et al. 2000).

Morphometric and biochemical data available so far provide compelling evidence of the importance of polyploidization in connection with differentiation, growth and aging of the normal organism, as well as with several pathological conditions, and evidence suggests that different genetic programs play a role in the expression of polyploidy in different cell lines and/or at different times in the life history of the organism. In connection with cell growth, also endoreplication should be considered (EDGAR and ORR-WEAVER, 2001). Anyhow, polyploid mononucleated cells of epithelial origin and mesenchymal multinucleated cells (sometimes called polykaryons: HEYMANN *et al.* 1998) should be regarded as different entities. In vitro studies have shown that several macro- and/or micromolecules may mediate multinucleated cell formation. So, the leukemia inhibitory factor (LIF), that is a pleiotropic cytokine known as inducer of acute phase protein synthesis in inflammation, can induce macrophage polykaryon formation from human bone marrow cultures (HEYNMANN *et al.* 1997), and human recombinant oncostatin M (OSM) displays the same effect (HEYNMANN *et al.* 1998). Other data suggest that 8-isoprostaglandin E2 (isoPGE2) enhances osteoclastic differentiation of marrow preosteoclasts (TINTUT *et al.* 2002). And so on...

CONCLUSIONS

The Discussion has been restricted to aspects inherent to the experimental section. However, the growing interest for the role of various cytokines and other regulators such as chromosomal passenger proteins, Aurora B, inner centromere proteins and Survivin (NAGATA *et al.* 2005) in the processes outlined in this paper may lead in the near future to better understand the mechanisms underlying polyploidy and its regulation.

ADDENDUM

When this manuscript was ready for submission, the 2006 July issue of Cytometry, Part A 69A, published two articles harmonizing with the experimental section of this paper. These articles, indeed, stress that it is important (i) to gain "the most detailed quantitative data from biological specimens" (TARNOK 2006) and (ii) to extract quantitative information from tissue by image analysis (VAN OSTA 2006). Subsequently, the 2007 January issue of Genomics published a paper elucidating by new methods the functional significance of multiple duplication of genome in somatic cells (ANATSKAJA AND VINOGRADOV 2007).

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