The Effects of Methotrexate on the Pancreas of Rats

— A Histochemical and Ultrastructural Study—

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ABSTRACT

Methotrexate is one of the well known anti-cancer chemotherapeutic agents and exerts its action by inhibiting mitoses by inhibition of nucleic acid synthesis. Its effect on actively proliferating normal and pathologic tissues have been well documented. However, little information is available on its effect on tissue which shows no active mitoses but does have a very active metabolic process, such as the pancreas. The present study investigated the histochemical and ultrastructural changes which take place within 24 hours after a single intraperitoneal injection of methotrexate.

Using a light microscope for observation, no specific or constant alteration was noted except for a mild acinar cell dissociation 18 hours after the injection. However, electron microscopic observations showed that several organelles of pancreatic acinar cell revealed ultrastructural changes such as vesiculation and dilation of the cisternae of endoplasmic reticulum, and the appearance of autophagic vacuoles which contained cellular organelles, and showed hyperplasia and dilation of Golgi complexes. The nuclei and zymogen granules were not significantly altered. The changes of endoplasmic reticulum were distinctly seen from 1 hour after the injection and were most severe at 6 hours. Autophagic vacuoles appeared at 6 hours and had progressively increased in number and size 18 hours after the injection.

Similar changes were also reported in experimental animals which were treated with several cytotoxic agents. According to this study, it is evident that a single administration of methotrexate within short time interval induced a series of ultrastructural alterations in several organelles of the pancreatic acinar cells.

It is not clear as yet whether or not this is a specific reaction of cells to methotrexate.

INTRODUCTION

Methotrexate is one of the well known folic acid antagonists. It is widely used as an anti-cancer chemotherapeutic agent. The mechanism of anticancerous action of this compound is based on the inhibition of mitoses by the interference of the enzyme, folic acid reductase, which catalyzes a necessary link in the series of reactions leading from folic acid to tetrahydrofolic acid or citrovorum factor. Tetrahydrofolic acid is involved in the transfer of one carbon units needed in the de novo synthesis of purines and pyrimidines and ultimately, nucleic acid. (Mead, 1963) Many studies have confirmed the inhibitory action of methotrexate on mitoses in actively proliferating normal and pathologic tissues, but little information is available on the morphologic effect of this compound on the tissue which shows
no active mitoses but is in an active metabolic process.

In 1969, Sohn reported the effects of methotrexate upon the exocrine glands of the pancreas in rats, and found that marked morphologic alterations were noted in animals, which were killed one day after one course of methotrexate (5 days with daily dosage of 0.3 mg/kg). The present study was undertaken to investigate the ultrastructural changes which take place within 24 hours after a single dose of methotrexate.

MATERIAL AND METHOD

Adult albino rats were used regardless of their sex. Average body weight was 239 gm ranging from 220 gm to 250 gm. Methotrexate (Lederle) was given intraperitoneally in a dose of 1.5 mg per kg of body weight. A couple of animals were killed at 1, 6 and 18 hours after the injection.

Tissue was removed while the animal was under ether anesthesia. For light microscopic study, one part of the tissue was fixed in 10 per cent formalin, embedded in paraffin, and hematoxylin, eosin, methylgreen pyronin, Feulgen reaction and modified tetrachrome stains (Fitzgerald, et al., 1968) were performed. For electron microscopic study, the other part was fixed in 1 per cent osmium tetroxide in 0.1M veronal buffer at pH 7.4. (Palade, 1952) The tissues were dehydrated with graded alcohol and embedded in Epon 812. Ultrathin sections were cut on a Porter Blum ultramicrotome with glass knife, and were stained with uranyl acetate and with lead hydroxide. (Reynolds, 1963) Sections were examined in the Hitachi HU 11E electron microscope.

RESULTS

Light Microscopic Study

The pancreas of the control rats were regularly lobulated, and the glandular cells were arranged in a well developed acinar structure, having a large amount of eosinophilic cytoplasm.

The nuclei were in a basal location. The cytoplasm adjacent to the nuclei revealed a moderate degree of basophilia with a hematoxylin eosin stain. Pyronophilic substances were distributed diffusely but were more numerous at the basal portion of the cell around the nuclei. Zymogen granules were abundant in amount and located at the apical portion of the cell.

The pancreas removed one hour after the intraperitoneal administration of methotrexate revealed no definite structural alterations with a hematoxylin-eosin stain. Although the pyronophilia was slightly reduced, the zymogen granules were still abundant. The pancreas removed 6 hours after methotrexate administration revealed a mild degree of acinar cell dissociation. The pyronophilia was equal in intensity as compared with the control pancreas. The zymogen granules were not reduced. At 18th hours, glandular cell dissociation was advanced without a notable decrease of pyronophilia. However, the zymogen granules were somewhat reduced in quantity. Throughout the experiment, no demonstrable changes were noted in the Feulgen reaction.

Electron Microscopic Study

The ultrastructural features of pancreatic acinar cells in control rats were in accord with the previous description. We will refer to the findings in the control rat only as they are in contrast to the experimental group. The fine structure of the control rat pancreas, as shown in Figure 1 did not differ from that reported previously. (Sjöstrand, 1961.; Palade, Siekevitz, and Caro, 1961)

In the experimental group, several structural alterations of organelles were found beginning 1 hour after the injection of methotrexate. However, some cells remained unchanged. Among the altered cells the degree of alteration varied considerably.

Nucleus: In control rat, the nucleus showed a round contour with relatively homogenous nucleoplasm. Experimental animals showed neither characteristic nor constant changes of the nuclei and nucleoli of cells.

Endoplasmic reticulum: The common findings in all experimental animals were segmental dilatation, of cisternae of granular endoplasmic reticulum, and;
segmentation of cisternae with the formation of rough-surfaced vesicles. The most severe dilatation of cisternae was noted in rats killed 6 hours after the injection (Fig. 3, 4)

**Golgi complex:** The Golgi complexes in control rats showed no notable changes. At 6 hours after the injection, cisternae were dilated. Dilation progressed further at 18 hours and was associated with an increase of small Golgi vesicles. (Fig. 3)

**Mitochondria:** At 6 hours, some of the mitochondria showed swelling and irregularity of the outer membranes. Mitochondria were frequently observed in autophagic vacuoles. Digested mitochondria showed distortion of shape, loss of cristae, and general swelling. (Fig. 3, 5)

**Autophagic vacuoles:** In the control rats, acinar cells contained a few small lysosomes but no significant autophagic vacuoles. In the experimental group, autophagic vacuoles were first noted 6 hours after the injection, and progressively increased in number and size until 18 hours. These vacuoles contained dense amorphous material or membranous debris, and showed relatively well preserved endoplasmic reticulum, ribosome, mitochondria, and zymogen granules. The autophagic vacuoles measured from 0.5 to 2.0 μ in diameter (Fig. 4, 5)

**Zymogen granules:** Zymogen granules persisted in the acinar cells of experimental rats until 18 hours. However, these granules were somewhat smaller than the usual zymogen granule in normal cells (Fig. 2, 6)

**Discussion**

Methotrexate exerts its antimetabolic effect by attacking some of the biosynthetic reactions related to folic acid metabolism. Mead (1963) stated that methotrexate could completely inhibit the formation of tetrahydrofolic acid by the enzyme system. In the absence of a folic acid antagonist, the tetrahydrofolic acid formed by the reductase can combine with the metabolically available one-carbon units to form various co-enzymes which are then used in biosynthetic reactions. One of the important biosynthetic reactions which utilizes tetrahydrofolate co-enzymes is the de-novo biosynthesis of purines. Another key biosynthetic reaction known to be inhibited by folic acid antagonists is the synthesis of thymidylate which in turn is essential for the synthesis of DNA. (Mead, 1963)

In our department, several experiments have been carried out to establish the morphologic effect of methotrexate on regeneration of the liver, wound healing, and the bone marrow (Song, 1966), on the placenta and fetus (Shin, 1967), on the gonads (Choo, 1963), on the gastrointestinal mucosa(Lee, 1968) and on the pancreas (Sohn, 1969). This is our first attempt to use the electron microscope to study the effect of methotrexate.

Pancreatic acinar cells, which serve a specific function of synthesis, storage, and secretion of a large amount of protein in the form of digestive enzymes, seem to be particularly susceptible to damage by compounds affecting protein synthesis and secretion. The effects of several such agents-ethionine (Ekkholm, et al., 1962), β-3-furorlanine (Hruban, et al., 1965), chloramphenicol and tetracyclin (Imai, 1966), actinomycin D (Rodriguez, 1967), puromycin (Longenecker, et al., 1968), aminocyclopentane carboxylic acid(ACPC, Chenard, and Auger, 1968)—on the pancreas have been studied ultrastructurally. However, there has been no available information about the morphologic effect of methotrexate on the pancreas.

In the pancreas, the high RNA content of pancreas formed part of the basis for the theory that RNA participates in protein synthesis, which was first enunciated by Caspersson (1941) and independently by Brachet (1942) about 28 years ago. Since the first suggestion, there has been enormous advance in our understanding of the role of RNA in protein synthesis in general. Allfrey, et al., (1963) showed that the rate of protein synthesis in endoplasmic reticulum of the mouse pancreas did not correlate well with the rate of RNA synthesis. Hokin and Hokin (1954) found in slices of pigeon pancreas that stimulation of enzyme synthesis by
providing the necessary amino-acids was not accompanied by any change in the RNA level nor by the incorporation of P³² into RNA. Hokin (1967) found that messenger RNA in the pancreas is quite stable and requires replenishment only very slowly.

Barton and Laird (1957) studied the effect of methotrexate on nucleic acid metabolism in mitotic and non-mitotic growth of the liver and the pancreas. They concluded that nucleic acid synthesis was inhibited only when the synthesis occurred at the time the cell was preparing to divide. Also, they concluded that RNA synthesis was not directly inhibited in vivo, but only indirectly, as a result of the inhibition of the preparation for mitosis. Recently Soln (1969) reported, however, that RNA synthesis and the subsequent enzyme-protein synthesis were inhibited by the administration of methotrexate, they observed the most severe morphologic changes 24 hours after the completion of one course of methotrexate.

It is evident from this study that a single administration of methotrexate induced a series of ultrastructural alterations in several organelles of the pancreatic acinar cells within a short time interval. The granular endoplasmic reticulum, the organelle concerned with synthesis of exocrine enzyme (Weiss, 1953; Palade, Sirclevitz, and Caro, 1961), showed vesiculation and dilatation of the cisternae, these changes were distinct within 1 hour after the intraperitoneal administration of methotrexate and were most severe 6 hours after the injection. These changes have been reported also in ethionine (Ekholm, et al., 1962) puromycin (Longnecker, et al., 1968) and aminocyclopentane carboxylic acid (ACPC)-treated rat (Chenard and Auger, 1968) and actinomycin D-treated mouse (Rodriguez, 1967) and were considered as a non-specific response to various kinds of injury. Watanabe (1955) reported that the vacuolar or vesicular forms were indicative of the inactive state of endoplasmic reticulum and Longnecker, et al., (1968) speculated that this alteration would be related to osmotic change. The matter is still not clear.

With puromycin (Longnecker, et al., 1968), ethionin (Ekholm, 1962) and β-3-furylaniln (Hruban, et al., 1965) a considerable number of intracisternal granules were reported. In contrast, no such granules were found in this experiment. These were considered to be structurally altered polypeptides (Longnecker, et al., 1968). If this speculation is true, such an alteration does not occur following methotrexate.

The increase in the number and size of autophagic vacuoles in the acinar cells was a significant progressive change. These vacuoles always contained the cytoplasmic organelles of the acinar cells. Similar changes were reported in pancreatic acinar cells in the puromycin, ethionin and actinomycin-treated animals. Hruban, et al. (1962), and Longnecker, et al., (1968) considered that these changes represent a common acinar cell reaction to injury, but Ekholm et al. (1962) considered that these represent aicinar cell reaction to a seriously modified protein synthesis, not to injury. The mitochondria of acinar cells have been understood to take no direct role in forming secretory substances, but rather to work indirectly as a supplier of the energy necessary for protein synthesis and, possibly, for its transport. (Imai, 1966) In this experiment, no significant morphologic alterations were noted.

Using autoradiography, Palade, et al., (1961) suggested that the direct participation of the nuclei in producing digestive enzymes was of little importance. In this experiment, no significant morphologic alterations of the nuclei were noted. Golgi complexes showed dilatation and hyperplasia 1 hour after the injection. Also these may reflect a disturbed storage of exocrine enzyme protein.

No significant decrease was noted in the quantity or quality of the zymogen granules. Although the time interval may be too short, this finding can not rule out the possibility that methotrexate inhibits enzyme-protein synthesis in the pancreas.

These ultrastructural changes may or may not represent a specific reaction of the pancreatic acinar cell to methotrexate, and they may be common
to other cytotoxic agents. However, it must be emphasized that some cells remained normal. Also, among the altered cells, the degree of deviation from the control cells varied considerably. Trier (1962) stated that the degree of cytoplasmic change may be related to 1) the status of ribonucleic acid (RNA) metabolism as well as the DNA synthetic activity of each cell at the time of exposure to the antimetabolite; 2) endogenous content of the enzyme folic acid reductase, which is inhibited by methotrexate; and 3) the quantities of preformed folic, dihydrofolic and tetrahydrofolic acid present in individual cells during exposure to methotrexate.

REFERENCES

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Fig. 1. A portion of acinar cells of the normal control rat. The acinar cell shows a nucleus (Nu), rough endoplasmic reticulum (ER) and mitochondria (Mi). The rough endoplasmic reticulum have regular arrangement with uniformly elongated cisternae. The ribosome particle (ri) is attached to the membranes of the cisternae. 30,000X

Fig. 2. Apical portion of pancreatic acinar cells of the control rat. Zymogen granules (Z) are abundant. Acinar lumen (Al), cell membrane (cm) are also evident. 12,000X
Fig. 3. A pancreatic acinar cells from rat killed 6 hours after methotrexate injection. The Nucleus (Nu) is large and sperical; Golgi complex (go) is greatly dilated and endoplasmic reticulum is dilated forming a rough-surfaced vesicle. 10,800X

Fig. 4. Portion of 4 acinar cells from rat killed 18 hours after the injection of methotrexate. Dilated golgi cisternae (v) and irregular sized autophagic vacuoles (ov) are present. Autophagic vacuoles contain mitochondria, a myeline-like material, and membranous debris. 22,000X
Fig. 5. Portion of acinar cell from same rat as shown in Fig. 4 with 2 large and one small autophagic vacuoles. Autophagic vacuoles (AV) contain irregularly shaped mitochondria, some granules, ribosome and membranous debris. Nucleus (Nu), mitochondria (Mi) and cell membrane (cm) are also evident. 30,000X

Fig. 6. Portion of an acinar lumen of a rat killed 18 hours after the injection. Zymogen granules are abundant near the acinar lumen (Al). 12,000X