AUTHORADIOGRAPHIC STUDIES ON THE DISTRIBUTION OF 14C-1,2-DIMETHYLHYDRAZINE DIHYDROCHLORIDE AND ITS EFFECT ON DNA SYNTHESIS IN SWISS MICE

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Light microscopic autoradiographic studies were made on the distribution of 14C-1,2-dimethylhydrazine dihydrochloride in Swiss mice and on the effect of 1,2-dimethylhydrazine dihydrochloride on DNA synthesis, using the 3H-thymidine incorporation technique. In the first study, 14C-1,2-dimethylhydrazine dihydrochloride was administered subcutaneously or orally. Large amount of silver grains were found in hepatocytes and substantially lower amount of silver grains observed in the endothelial cells and epithelial cells of colon. In the second study, repeated injections or oral administrations of 1,2-dimethylhydrazine dihydrochloride were given to mice which subsequently received 3H-thymidine treatment. A somewhat higher amount of thymidine incorporation in DNA was noted in the epithelial cells of the colon of subcutaneously and orally treated mice at two occasions and a substantially higher amount in the endothelial cells of blood vessels in liver of mice treated by both routes than in the corresponding controls. In three instances, however, the amount of incorporation decreased; in the hepatocytes and endothelium at 1 week and 24 hr, respectively, after oral treatment, and in the epithelium of the colon at 3 months, after subcutaneous administration.

In the mice treated with 1,2-dimethylhydrazine dihydrochloride, a significantly high amount of 3H-thymidine incorporation was observed in the endothelial cells of blood vessel in liver from which tumors later arose, and somewhat high in the hepatocytes in which tumor did not develop. In the epithelial cells of colon, no apparent relationship can be seen between these events. No association was seen in the distribution of 14C-1,2-dimethylhydrazine dihydrochloride and tumor development in various cells.

In earlier carcinogenesis studies using 1,2-dimethylhydrazine dihydrochloride in 3 species, mice, hamsters, and rats, no tumors were observed in the vascular system.6,12,16,17,20 In fact, in some of these the compound failed to produce any tumors,12 while in others tumors of the intestine, lungs, and liver were elicited by repeated subcutaneous administrations of this chemical. Recently, however, it was reported that 1,2-dimethylhydrazine dihydrochloride, when given orally at low dosages, induced blood vessel tumors in rats,7 and vascular and lung tumors in mice.19

The present study is concerned specifically with (a) the localization and distribution of 14C-labeled 1,2-dimethylhydrazine dihydrochloride and tumor development in various cells.

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chloride given subcutaneously or orally and (b) the effect of 1,2-dimethylhydrazine dihydrochloride administered as in Group 1, on DNA synthesis, using ³H-thymidine incorporation. The two different treatment routes were necessitated by the fact that earlier, when this compound was given subcutaneously, it induced mainly tumors of the intestines, lungs, and blood vessels, but when administered orally, it produced only vascular and lung tumors.19)

MATERIALS AND METHODS

Swiss albino female mice from the colony randomly bred by us since 1951 were used. They were housed in plastic cages with granular cellulose bedding and given Wayne Lab-Blox diet in regular pellets (Allied Mills, Inc., Chicago) and tap water or the test solution freely.

The chemicals used were symmetrical 1,2-dimethylhydrazine dihydrochloride (1,2-dimethylhydrazine), obtained from K and K Laboratories, Inc., Plainview, N.Y., symmetrical ¹⁴C-labeled 1,2-dimethylhydrazine dihydrochloride (¹⁴C-1,2-dimethylhydrazine), with a specific activity of 0.899 mCi/mmol, and tritiated thymidine, with a specific activity of 6.7 Ci/mmol, both from the New England Nuclear, Boston, Mass.

The treatments and the experimental and control groups are briefly described as follows:

Group 1: Forty-five 8-week-old female mice received a single subcutaneous injection of 20 µg/g body weight of ¹⁴C-1,2-dimethylhydrazine. Five mice were sacrificed at each of the following time intervals: 1, 3, 6, 12, and 24 hr, 4 days, 1 and 2 weeks, and 1 month after injection.

Group 2: Forty-five 8-week-old female mice were given orally by gavage a single dose of 500 µg of ¹⁴C-1,2-dimethylhydrazine. Five mice were sacrificed at the same time intervals as in Group 1.

Group 3: Thirty 8-week-old female mice received 0.001% 1,2-dimethylhydrazine in the drinking water daily. Five mice were sacrificed at each of the following time intervals: 24 hr, 1 week, and 1, 3, and 6 months after beginning of treatment. One hour before sacrifice each mouse was given 1 µCi/g body weight of ³H-thymidine intraperitoneally.

Group 4: Thirty 8-week-old female mice were treated with ten weekly subcutaneous injections of 20 µg/g body weight of 1,2-dimethylhydrazine. Five mice were sacrificed at 24 hr, 1 week, and 1 and 3 months after the last treatment. One hour before sacrifice each mouse was given 1 µCi/g body weight of ³H-thymidine.

Group 5: As controls, an appropriate number of untreated mice were sacrificed for Groups 1 and 2 at the same time intervals. Also for Groups 3 and 4 an appropriate number of untreated animals were kept and sacrificed at similar time sequences. These latter groups received one hour before killing, 1 µCi/g body weight of ³H-thymidine.

Pieces of liver and colon tissues from mice in Groups 1, 2, 3, and 4, plus the corresponding control mouse tissues were fixed in 10% buffered Formalin for 48 hr. Then these tissues were dehydrated, embedded, and cut less than 4 µm in thickness. Later, they were deparaffinized with several changes of xylene followed by rinsing in ethanol and finally in water. Autoradiographs were prepared by a modified dipping technique. NTB-3 Kodak Nuclear Track Emulsion (Eastman Kodak Co., Rochester, N.Y.) was used. Then the slides were stored in light-tight boxes containing Drierite and kept at 4°C in a refrigerator for 15, 30, 45, 60, and 90 days. The slides exposed were developed by Kodak D-19 Developer at 20°C for 1 min, washed for 20 sec in distilled water, and finally fixed in Kodak Acid Fixer for 5 min. After rinsing with tap water for 5 min, the slides were stained with Mayer’s Hematoxylin and Eosin. The control slides were identically processed as the treated ones.

The labeled and unlabeled cells were counted microscopically on autoradiographic histological specimens in a total number of at least 500 cells of an animal in the ¹⁴C-labeled experiments and 1000 cells of an animal in the ³H-thymidine experiments in the fields chosen randomly. Five animals were counted respectively. The mean±SD evaluation was made according to a widely used method in biology.

RESULT

In the animals treated with a single injection of ¹⁴C-1,2-dimethylhydrazine, the number of silver grains was examined in the hepatocytes, endothelial cells of blood vessels in liver and epithelial cells of colon. In each tissue 500 cells were counted. Fig. 1 shows the results in the hepatocytes in which a large amount of silver grains were observed at the beginning, which steadily diminished. Fig. 2 presents the data in the endothelial cells of blood vessels in the liver. It is clear that a definitely lower amount of silver grains were
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Fig. 1. Silver grains in hepatocytes after the application of $^{14}$C-1,2-dimethylhydrazine

Fig. 2. Silver grains in endothelial cells of blood vessels in the liver after the application of $^{14}$C-1,2-dimethylhydrazine
found, which started to decrease after 6 hr
and, by the 4th day, its level reached the
minimum. In Fig. 3, the amount of silver
grains found in the epithelium of colon is
illustrated and it is obvious that it was similar
to the level found in the endothelial cells;
however, the peak was reached 3 hr after
treatment followed by a slow decrease. It is
also clear from Figs. 1~3 that there are some
differences in the amount of silver grains in the
subcutaneously and orally treated animals.

Fig. 3. Sliver grains in epithelial cells of colon after the application of 14C-1,2-
dimethylhydrazine

Fig. 4. Labeled hepatocytes
Left: 1,2-Dimethylhydrazine orally followed by 3H-thymidine.
Right: Ten subcutaneous injections of 1,2-dimethylhydrazine followed by 3H-thymidine.
In the mice treated repeatedly with 1,2-dimethylhydrazine and followed by 3H-thymidine injection, the DNA synthesis was studied by determining the labeled hepatocytes, endothelial cells of blood vessels of liver, and epithelial cells of colon. In each tissue at least 1000 cells were counted. As it can be seen from Fig. 4, the number of labeled liver cells of the orally treated mice were higher at 1 week (P<0.01) and at 3 and 6 months (P>0.1) from that of the subcutaneously treated animals at 1 and 3 months (P<0.02 and P<0.1, respectively) after the application of 1,2-dimethylhydrazine than in the control groups. However, in the hepatocytes of the orally treated mice a decreased rate of incorporation was found at 1 week after the application of 1,2-dimethylhydrazine. As can be seen in Fig. 5, in the endothelial cells of blood vessels in the liver of the orally treated mice a substantially higher rate of incorporation was found at 24 hr (P<0.02), 1 week (P<0.01), and 1 month (P<0.05) after treatment. In contrast, in the endothelial cells of the orally treated mice, the rate of thymidine incorporation decreased 24 hr after 1,2-dimethylhydrazine treatment. It
is apparent from Fig. 6 that in the orally treated animals the labeling in the colon epithelium was only significantly higher at 1 week (P<0.1) after the administration of 1,2-dimethylhydrazine than in the corresponding controls. Also, a definitely higher rate of incorporation was noted in the epithelial cells of colon of mice treated once with 1,2-dimethylhydrazine and killed 3 hr later, the silver grains were also evident (Photo 3). Indeed very few silver grains were recognizable in the endothelial cells of liver in identically treated mice which were killed 1 month later (Photo 4). In the epithelial cells of colon of mice treated once with 14C-1,2-dimethylhydrazine and killed 3 hr later, many silver grains were observed (Photo 5). Very little silver grains were visible in the epithelial cells of colon of identically treated mice which were killed 1 month after treatment (Photo 6).

In the liver of mice treated orally with 1,2-dimethylhydrazine for 24 hr and followed by 3H-thymidine injection few labeled hepatocytes were seen (Photo 7). The thymidine incorporation also concentrated at the nucleus region of these cells (Photo 8). In the endothelial cells of blood vessels in liver of mice treated orally with 1,2-dimethylhydrazine for 24 hr and followed by 3H-thymidine injection, the higher magnification photomicrograph of few cells which showed labeled markings exhibits the concentration of grains in the nucleus region (Photo 9). In the endothelial cells of blood vessels in liver of mice treated with 1,2-dimethylhydrazine orally for 1 month followed by 3H-thymidine injection, the higher magnification microphotograph shows that the silver grains concentrated at the nucleus region of these cells (Photo 10). Photo 11 shows the markings in many epithelial cells mainly in the lower portion of crypts in the colon. This mouse was treated with 1,2-dimethylhydrazine orally for 24 hr followed by 3H-thymidine injection 1 hr before death. After 3 months of
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oral treatment with 1,2-dimethylhydrazine and by subsequent $^3$H-thymidine injection the number of labeled cells decreased in the colon (Photo 12).

**DISCUSSION**

The present findings first demonstrate the distribution of $^{14}$C-labeled 1,2-dimethylhydrazine in the hepatocytes, endothelial cells of blood vessels in liver, and epithelial cells of the colon in Swiss mice treated orally or subcutaneously. The silver grains were determined by light microscopic autoradiography at 1, 3, 6, 12, and 24 hr, 4 days, and at 1, 2, and 4 weeks after administration of the carcinogen. The highest amount of silver grains were recorded in the hepatocytes, while both the endothelium of blood vessels in liver and epithelium of the colon showed a substantially lower amount of silver grains. Interestingly, the highest amount of silver grains was found 1 hr after administration of the chemical in the hepatocytes and endothelial cells of blood vessels in liver, but 3 hr after treatment, in the epithelial cells of the colon. Subsequently, in all instances, the amount steadily decreased. By taking into account the results of long-term tumorigenesis studies, it is obvious that no correlation can be seen between the distribution of $^{14}$C-labeled 1,2-dimethylhydrazine or its breakdown products and tumor development in these particular cells. The study secondly shows the effect of 1,2-dimethylhydrazine administered orally and subcutaneously on DNA synthesis, using the $^3$H-thymidine incorporation technique. The number of cells labeled with $^3$H-thymidine was examined in the hepatocytes, the endothelium of blood vessels in liver, and the epithelium of colon at 24 hr, 1 week, and 1 and 3 months (and in some cases, 6 months) after the administration of 1,2-dimethylhydrazine. These results indicate that insofar as the total number of labeled cells is concerned, the highest number was seen in the colon epithelium, fewer in the endothelial cells of blood vessels in liver, and even smaller number in the hepatocytes. The interpretation of findings in the colon is difficult, because a high number of labeled cells was found at 1 month after the application of carcinogen in mice treated by 10 subcutaneous injections. Also, in the orally treated mice, the number of labeled epithelial cells of colon was high at 1 week after the administration of 1,2-dimethylhydrazine. Yet, a high incidence of colonic tumors was observed in the subcutaneously treated but none whatsoever in the orally treated mice. 1,2-Dimethylhydrazine given by both routes induced blood vessel tumors, and the number of labeled cells in the endothelium of blood vessels in liver was significantly higher in both groups than in the controls. Therefore, a correlation exists between these two events. Finally, the number of labeled hepatocytes was only slightly higher in the treated than in the control groups. As a matter of fact, no liver cell tumor was observed in the long-term studies. Nevertheless, it should also be mentioned that in a few cases the amount of thymidine incorporation in DNA decreased in the 1, 2-dimethylhydrazine-treated mice. These included the hepatocytes and endothelial cells of blood vessels in liver at 1 week and 24 hr after oral treatment respectively and the epithelial cells of the colon at 3 months after subcutaneous administration of 1,2-dimethylhydrazine.

It has been known for some time that various cancer-producing agents such as the X-ray and chemicals, inhibit and later enhance DNA synthesis.1,8) The initial depression of DNA synthesis was demonstrated after applications of urethan18) and 7,12-dimethylbenz-[a]anthracene.10) Even though a number of tumor-producing compounds inhibited the DNA synthesis, it is also true that other non-tumor producing chemicals, including Actinomycin-D, acted likewise.2,10) The chemical carcinogens at the same time also exerted a strong toxic action. Therefore, it is equally logical to suppose that their inhibition of the synthetic cell functions could have represented a toxic side effect. It may be worthwhile
also to note here that the normal physiological cell functions were also shown as affecting the synthesis of nucleic acids. It was demonstrated earlier, in the surface area of human colonic epithelial cells and in mucosa undergoing hyperplasia, that increased incorporation of 3H-thymidine in DNA occurred.\(^5\) Human villous papilloma and colonic carcinoma cells exhibited a decreased rate of incorporation of 3H-thymidine into DNA, by comparison with adjacent colonic tissue.\(^11,13\)

Thus it appears that our results are partially in agreement with the findings of previous studies concerning the actions of tumorigenic chemicals on DNA synthesis. Certainly it appears that additional studies are needed to clarify this line of inquiry.

Investigators in the past also tried to determine the localization of radioactive compounds in the skin of animals and to speculate whether a correlation exists between the distribution of chemicals and tumor development in those specific cells. For this reason they used labeled compounds such as \(^{14}C\)-7,12-dimethylbenz[a]anthracene\(^3\) and \(^{3}H\)-7,12-dimethylbenz[a]anthracene,\(^15\) and observed their concentration in the target tissues. Since these workers were essentially interested in checking the cells from which subsequent tumors developed, they apparently paid little or no attention to other tissues and cells from which tumors did not arise. The findings of our present study obviously do not correlate with the results of the lifelong tumorigenesis investigation because of, for instance, the silver grains concentrated in the hepatocytes from which tumors did not develop. Therefore, it is hoped that additional studies will be conducted, which may throw some light on this research area.

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REFERENCES

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EXPLANATION OF PLATES

Photo 1. Liver. All cells contain large amount of grains. Female mice, 8 weeks old, treated once with \(^{14}C\)-1,2-dimethylhydrazine subcutaneously and killed 1 hr after treatment. Hematoxylin and Eosin. \(\times 720\).

Photo 2. Liver. Few silver grains are visible in the hepatocytes. Female mice, 12 weeks old, treated similarly as shown in Photo 1, and killed 1 month after treatment. Hematoxylin and Eosin. \(\times 720\).

Photo 3. Endothelium of blood vessel in liver. Observe the silver grains (arrow) in an endothelial cell. Female mice, 8 weeks old, treated once
with $^{14}$C-1,2-dimethylhydrazine subcutaneously and killed 3 hr after treatment. Hematoxylin and Eosin. $\times 720$.

Photo 4. Endothelium of blood vessel in liver. Very few silver grains (arrows) are visible in these cells. Female mice, 12 weeks old, treated similarly as the animal shown in Photo 3 and killed 1 month after treatment. Hematoxylin and Eosin. $\times 720$.

Photo 5. Epithelium of colon. The silver grains are recognizable in various cells. Female mice, 8 weeks old, treated once with $^{14}$C-1,2-dimethylhydrazine orally and killed 3 hr after treatment. Hematoxylin and Eosin. $\times 720$.

Photo 6. Epithelium of colon. The cells contain few grains. Female mice, 12 weeks old, treated similarly as shown in Photo 5 and killed 1 month after treatment. Hematoxylin and Eosin. $\times 720$.

Photo 7. Liver. Two labeled hepatocytes in the area; one is a dividing cell. Female mice, 8 weeks old, treated with 1,2-dimethylhydrazine orally for 24 hr followed by $^3$H-thymidine injection. Hematoxylin and Eosin. $\times 150$.

Photo 8. Liver. Note in the center the highly labeled nucleus of a hepatocyte. Same as shown in Photo 7. Hematoxylin and Eosin. $\times 720$.

Photo 9. Endothelium, liver. A labeled endothelial cell of blood vessel (arrow) is distinct. Female mice, 8 weeks old, treated similarly as Photo 7. Hematoxylin and Eosin. $\times 720$.

Photo 10. Endothelium, liver. Two endothelial cells of blood vessel (arrows) are showing heavy concentration of silver grains in their nuclei. Female mice, 12 weeks old, treated with 1,2-dimethylhydrazine orally for 1 month followed by $^3$H-thymidine injection. Hematoxylin and Eosin. $\times 720$.

Photo 11. Colon. Many labeled epithelial cells are visible, mainly in the lower portion of crypts. Female mice, 8 weeks old, treated with 1,2-dimethylhydrazine orally for 24 hr followed by $^3$H-thymidine injection. Hematoxylin and Eosin. $\times 150$.

Photo 12. Colon. The labeled cells are fewer than in the previous picture. Female mice, 20 weeks old, treated with 1,2-dimethylhydrazine orally for 3 months followed by $^3$H-thymidine injection. Hematoxylin and Eosin. $\times 150$. 

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