Topical Skin Applications of Cantharidin and Asiaticoside Reticuloses and Epidermal Tumors in Hairless Mice after

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SUMMARY

Cantharidin, a vesicant agent, and asiaticoside, which promotes the healing of skin ulcers, were tested for carcinogenicity by topical applications to the skin of hairless mice. A short-term tetrazolium test indicated that both compounds were weak carcinogens. The compounds were then separately painted twice weekly on the dorsal skin up to about 20 months; some of the mice had previously been initiated with a small dose of 20-methylcholanthrene (MCA). A control group which received only the solvent benzene after MCA initiation was also studied.

Cantharidin acted as a weak but complete carcinogen on the skin, causing carcinomas in 6.3% of the animals. These carcinomas did not appear before about 16 months of observation. Before this, the painted, MCA-initiated animals had a significantly lower number of papillomas of the skin than the corresponding control group with only benzene treatment. It therefore seemed that cantharidin in the early stages of painting was tumor inhibitory, or "anticarcinogenic," possibly due to selective damage of latent tumor cells.

By systematic autopsy it was found that nearly 60% of the MCA-initiated, cantharidin-painted mice had reticuloses or malignant lymphomas, while only about 30% of the corresponding MCA-initiated, benzene or asiaticoside-treated animals had such neoplasms. It is therefore concluded that cantharidin penetrates the skin and promotes the development of tumors in the reticuloendothelial system.

Asiaticoside dissolved in benzene gave an increased yield of papillomas and also 2.5% skin sarcomas of the animals, indicating an effect on the dermis as well.

INTRODUCTION

The vesicant cantharidin produces severe damage to the skin. In the hairless mouse epidermis small doses provoke acute cell death and cell loss with subsequent regenerative hyperplasia (10, 25). Although the compound is generally considered to be noncarcinogenic, it has been found in some studies to promote tumors in previously initiated skin (12, 22) and a few papillomas when applied together with croton oil (23).

Asiaticoside (Madécassol), a glycoside terpene from the plant Centella asiatica, is used in medicine to improve healing of wounds and chronic ulcers (16, 19, 27). The compound has a low toxicity and a few applications do not produce much change in the skin (17, 18). Still, due to the cicatrization and growth-stimulating effect on the skin which has been reported, we thought that carcinogenic properties might become apparent after repeated applications.

We therefore considered a more thorough examination of both compounds using short-term as well as long-term application tests to be necessary. With the tetrazolium test (13), carcinogenic properties of chemical substances can be indicated within a few days after a single application to the skin. The test is based on the observation that carcinogens damage the cells in such a way that the reduction of tetrazolium to formazan in the cell is increased by more than 20%. As with other short-term tests, the tetrazolium test is not completely specific, and sometimes it is invalid for weak carcinogens.

MATERIALS AND METHODS

Hr/hr hairless mice 8 to 10 weeks old were kept 5 in each cage and given a standard diet and continuous water supply. The rate of the formazan deposition in epidermis was measured photometrically 1 and 2 days after a single application of each compound, and the values were expressed as ratio of painted to unpainted skin. Noncarcinogenic irritants and the solvent benzene generally give a reduction or only a slight increase in the rate of formazan deposition (13).

The long-term studies consisted of applications on the dorsal skin by a fine brush twice weekly. The painting was performed regularly during the entire lifetime of the animals up to 2 years. Systematic autopsies with histological examination of the skin as well as internal organs were performed routinely. The reticuloses were typed according to the nomenclature of Chouroulinkov et al. (5). The histological criteria for reticuloses are mentioned under "Experiments and Results."

Cantharidin (Nutritional Biochemicals Corp., Cleveland, Ohio) and Asiaticoside (Laroche-Navarrotte, Lecallois-Paris, Seine, France) dissolved in benzolum liquid pro analysisi (Riedel-De Haën AG, Hannover, Germany) were used for painting. Some of the mice were previously initiated with 0.1 ml 0.1% MCA (L. Light and Co., Colnbrook, England) 2 weeks prior to the start of the regular paintings. This dose is by itself not sufficient to produce cancers of the hairless mouse skin, but some of the animals get 1 or more papillomas during an observation time of 18 months (14).

Received November 29, 1971; accepted March 29, 1972.

1 The abbreviation used is: MCA, 20-methylcholanthrene.
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Table 1

Tumor yield after application of cantharidin asiaticoside and the solvent benzene

The percentages are expressed per number of animals alive at appearance of the 1st tumor.

<table>
<thead>
<tr>
<th></th>
<th>No. of mice</th>
<th>Mean life-span (mo.)</th>
<th>% animals with skin tumors</th>
<th>% animals with malignant skin tumors</th>
<th>Total % with tumors</th>
<th>% internal</th>
<th>% reticuloses and malignant lymphomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cantharidin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCA-initiated</td>
<td>42</td>
<td>19.1</td>
<td>59.5</td>
<td>1.1</td>
<td>80.9</td>
<td>63.4</td>
<td>56.1</td>
</tr>
<tr>
<td>Alone</td>
<td>32</td>
<td>18.8</td>
<td>31.3</td>
<td>6.3</td>
<td>60.3</td>
<td>51.6</td>
<td>28.1</td>
</tr>
<tr>
<td>Asiaticoside</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCA-initiated</td>
<td>29</td>
<td>18.5</td>
<td>72.4</td>
<td>3.4</td>
<td>82.7</td>
<td>44.0</td>
<td>28.0</td>
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<tr>
<td>Alone</td>
<td>28</td>
<td>21.2</td>
<td>60.7</td>
<td>2.5</td>
<td>62.1</td>
<td>50.0</td>
<td>36.4</td>
</tr>
<tr>
<td>Benzene, MCA-initiated</td>
<td>34</td>
<td>19.8</td>
<td>55.9</td>
<td>2.9</td>
<td>64.7</td>
<td>34.3</td>
<td>25.0</td>
</tr>
</tbody>
</table>

EXPERIMENTS AND RESULTS

Cantharidin was tested with the tetrazolium method at concentrations of 0.032 and 0.016%. Higher concentrations often produce ulcers, and the 0.125% solution gave complete acantholysis and necrosis of hairless mouse skin. For repeated applications 0.016% was the highest concentration that could be used without development of ulcers in the skin.

Asiaticoside was used at a concentration of 0.10% in benzene. The solution had to be shaken thoroughly before use to avoid precipitation. Asiaticoside did not produce necrosis or acantholysis of the skin and did not appear to be toxic.

The tetrazolium test was performed with groups of 8 mice for each compound. Both compounds gave values for formazan production which were indicative of carcinogenic potency. Mean ratio of painted to unpainted skin was 1.48 and 1.25 after 0.032 and 0.016% cantharidin, respectively, and 1.27 after asiaticoside painting.

Long-Term Studies with Repeated Applications

Thirty mice, 15 of each sex, were painted twice weekly with 0.032% cantharidin, altogether 20 times, and thereafter observed for the rest of their lifetime up to 19 months. One single papilloma of the back skin was observed after 12 months. Otherwise, some hyperpigmentation and minor ulceration of the skin was seen during the 10 weeks of painting. Autopsies were not performed in this group.

In Group B, 42 mice were initiated with MCA, and 14 days later twice weekly paintings with 0.016% cantharidin were started.

In Group C, 32 mice were painted with 0.016% cantharidin twice weekly.

In Group D, 29 mice were initiated with MCA, and 14 days later twice weekly paintings with 0.1% asiaticoside were started.

In Group E, 28 mice were painted with asiaticoside twice weekly.

In Group F, 34 mice were initiated with MCA, and 14 days later twice weekly paintings with the solvent benzene were started.

In Groups B to F, paintings were performed for the rest of the lifetime of the animals. Equal numbers of males and females were used in each group. The results are shown in Table 1 and Charts 1 and 2. "Number of mice" a represents animals alive at appearance of the 1st tumor. Some of the animals succumbed from intercurrent diseases before the tumors started to develop. At the end of the experiments the mice were very old and many died. For this reason the total observation time could not be identical in the different groups.

Skin Cancer. From Table 1 it is seen that 0.016% cantharidin dissolved in benzene acted as a complete carcinogen and gave squamous carcinomas in 6.3% of the animals. Benzene in itself is not carcinogenic to the skin and caused in a later-performed control group some very small, benign papillomas in 7.3% of the animals during the same observation time (Chart 1). Painting with cantharidin gave the same yield of carcinomas both with and without MCA initiation, namely 7.1 and 6.3%, respectively. The corresponding yield with benzene painting after MCA treatment was 2.9%, or 1 carcinoma in 34 animals. A later control series with benzene painting alone gave no carcinomas. After asiaticoside painting, the tumor yield was 3.4% with MCA initiation and 2.5% without, or 1 malignant tumor in 29 mice for MCA and 1 in 28 mice without MCA, respectively. Both these tumors were sarcomas of the dermis. No carcinomas were found.
Skin Papillomas. When all papillomas of the skin were included, the picture was different. The histological types were verrucous hyperplasias and sebaceous warts.

In the control group with benzene painting after MCA initiation, the number of mice with papillomas increased rapidly from the 6th to the 14th month and then gradually declined.

In contrast, the corresponding group with cantharidin in benzene after MCA initiation showed a slightly slower increase and contained a significantly lower number of mice with papillomas until the 18th month. \( p < 0.05 \) in a one-tail \( \chi^2 \) test. By the 20th month, the number was equal in the 2 groups again. Finally, at 22 months the numbers of mice with papillomas increased rapidly. No malignant tumors of the skin appeared before the last 4 to 5 months of observation.

In the MCA-initiated asiaticoside group, the number of mice with papillomas was higher than the control group with benzene during the entire observation period (Chart 2). The last observations were significantly higher than that of the control group \( (p < 0.05) \).

Without initiation, the number of mice with papillomas increased more slowly than but parallel with the initiation groups both after cantharidin and after asiaticoside painting (Charts 1 and 2). Asiaticoside produced the highest tumor yield, at 64.2% and cantharidin produced 34.4%, while benzene alone produced only 7.3%.

In the MCA-initiated groups, the average number of papillomas per mouse was slightly higher after painting with benzene than with the other compounds. The number of papillomas was, however, variable from mouse to mouse, especially in the benzene group, ranging from 1 to 22. The average numbers may therefore be misleading and are not shown in the tables.

Internal Tumors. A relatively high incidence of internal tumors was found in all the groups studied (Table 1). These were mostly reticuloses and malignant lymphomas. Painting with cantharidin on MCA-initiated skin resulted in an incidence of 56% of these tumors, while with the control group with benzene after initiation there was only 25% incidence. Painting with cantharidin alone was accompanied with 28% reticuloses.

After asiaticoside painting, 28% reticuloses and malignant lymphomas were seen in the MCA-initiated group, and 36% were seen in the noninitiated group. None of these numbers were significantly different from the MCA-initiated benzene control group.

The frequency of other internal tumors was about the same in all the groups; the distribution of the different histological types is given in Table 2.

In the investigated hairless mice, the lymphoreticulosis (or reticuloendotheliosis) was by far the most common type, with 56% of the total. The other types, reticulogranulomatosis, reticuloendotheliosis and reticulolymphogranulomatosis (Hodgkin's type) constituted from 8 to 14% each. Some of the characteristic histological types are shown in Figs. 1 to 3.

The typing of reticuloses is difficult because the border between a strong hyperplasia and a reticulosis is not very sharp. In addition, the group reticuloses includes a variety of histological patterns from possible benign lesions to frankly malignant ones with invasive growth. On the other hand, the distinction between malignant lymphomas and reticuloses is based partly on pure histological judgment and partly on the fact that a lymphoma is generally more localized, while reticuloses are generalized lesions. The typing in this study has been performed in collaboration with Dr. I. Chouroulinkov and Dr. M. Guérin to ensure constant histological criteria and nomenclature. In addition, one of us typed all the reticuloses blindly, with a good correspondence in typing.

DISCUSSION

Quite recently, it has been stated that hairless mice are more refractive to skin carcinogenesis than the corresponding hair-possessing strain (11). On the other hand, hairlessness is a recessive genetic characteristic that can be introduced into any
strain of mice, be they tumor resistant or tumor sensitive. This study demonstrates that the strain of hairless mice kept at our institute in Oslo is sensitive even to substances with very weak carcinogenic potency, provided there is a sufficiently long observation period.

**Cantharidin.** A limited amount of applications of cantharidin will not produce tumors in the skin, even after a long observation time (20, 21, 24), and the substance has even been reported to inhibit tumor development initiated by carcinogenic tar (1). Our results with 0.032% solution (Group A) were in accordance with these reports; but cantharidin has also been reported to promote tumors in the epidermis, first by Pound and Withers (22) and later by Hennings and Boutwell (12). Roe and Salman obtained 6 skin papillomas on 4 animals after painting a group of 20 animals with a total dose of 0.63 mg combined with croton oil applications. In our long-term paintings, cantharidin dissolved in benzene acted as a weak but complete carcinogen on the skin, producing a few carcinomas (Group C). This carcinogenicity had previously been indicated by the tetrazolium method.

One striking feature of cantharidin was that in the MCA-initiated group the number of animals with papillomas was significantly lower during the 1st 18 months than that of the corresponding control group with solvent painting (Chart 1). Then the number in the cantharidin group increased rapidly, while the increase in the benzene group was declining. In other words, cantharidin inhibited the development of papillomas during the 1st three-fourths of the observation period. If the investigation had stopped at this stage, the conclusion would be, as was that of Berenblum (1), that cantharidin is “anticarcinogenic.” However, during the rest of the observation period, the number of papillomas reached above the control group and several skin carcinomas occurred, meaning that cantharidin not only promoted tumors but is also a weak but complete carcinogen.

This feature is in accordance with other studies of the so-called “anticarcinogenesis.” It has been demonstrated that 2 compounds that are individually strong carcinogens may produce no tumors when they are administered together or with a short time interval. This means that one carcinogen may inhibit the tumor production by another carcinogen. Possible mechanisms for this has been extensively studied by other workers (28).

In the light of this it appears that cantharidin is able temporarily to inhibit the development of papillomas during benzene painting on mice previously subjected to MCA initiation. This may be due to a strong toxic effect of cantharidin upon initiated cells; it killed cells which otherwise would become precursors to tumor cells. The toxic effect of cantharidin upon hairless mouse epidermis has been demonstrated by Skjaeggestad (25), who found a cell loss of similar magnitude to that found after treatment with strong carcinogens. Other irritants like mustard oil did not show this effect, but only a small cell loss. With repeated applications of 0.032% cantharidin in benzene, Elgio (10) observed waves on intense proliferation in the epidermis with development of transient hyperplasia.

As well as having a weak carcinogenic potency on the skin, cantharidin seemed to be resorbed through the epidermis and seemed strongly to affect the reticuloendothelial system. This effect appeared to be general, since all kinds of reticuloses and malignant lymphomas from different organs were observed. These tumors also occur spontaneously from time to time in our hairless mice, but always in small numbers. In the MCA-initiated group with cantharidin painting, as many as 56% of the animals developed reticuloses and malignant lymphomas.

On the other hand, painting with cantharidin alone did not give higher yield of this type of tumors than the MCA-initiated, benzene-painted control group, the yield being 28 and 25%, respectively. It therefore appears that cantharidin acts as a promoter of reticuloses after previous MCA initiation.

Here we have no such qualitative carcinogenic effect as in the skin, where carcinomas do not occur spontaneously. Instead, we have a quantitative, possibly unspecific carcinogenic effect. In other words there was a very high yield of such tumors as are also seen with lower frequency in untreated hairless mice of advanced age.

**Asiaticoside.** Thiers et al. (26) reported that asiaticoside improved the healing of skin wounds by increasing the formation of connective tissue. In contrast, Lawrence (18) found that the drug did not reduce the mortality or influence the progress of healing in mice with experimental burns. It was not very toxic when compared with other therapeutic compounds. However, when injected s.c. a marked hyperplasia of the epidermis occurred after 2 days.

In our experiments hyperplasia of the skin was seen after a few applications of asiaticoside; but this was probably due to the solvent benzene, which also provokes hyperplasia after repeated applications.

To our knowledge carcinogenesis studies have never been performed with asiaticoside. The tetrazolium test indicated a possible carcinogenic potency on the skin, and our long-term paintings caused one sarcoma of the dermis in each group. A number of papillomas in the epidermis was also observed, and this was considerably higher than that seen after painting with the solvent both with and without MCA initiation. The incidence of reticuloses and malignant lymphomas was higher in the asiaticoside-treated mice than in the controls, but the difference was not significant.

Asiaticoside can therefore be classified as a weak tumor promoter in the hairless mouse epidermis. It also seems to be very weakly carcinogenic to the dermis by surface application to the skin. This is an interesting feature in connection with earlier reports that the compound mainly works on the dermis (26, 27).

Repeated applications over a very long period were needed for asiaticoside to show a weak promoting and possibly carcinogenic effect in the skin of hairless mice. We therefore do not know whether it can be carcinogenic to other organs and to other species. Inasmuch as the compound is used for skin application in human medicine, a strong carcinogenic effect in our mice would have been alarming. However, at the present moment there are no reports on such harmful effects of the drug on the skin, and further tests should be performed.

**Benzene.** Benzene has never been shown to be carcinogenic to the skin (15), although repeated applications cause epidermal hyperplasia (10) and long-term treatment may induce some few papillomas (Chart 1). The one carcinoma that we observed in the MCA-initiated mice (Group F) may be due
to the very weak promoting power of benzene but may equally well have occurred by chance.

On the other hand, benzene is known as a leukemogenic agent in both humans and animals, possibly because after administration the concentration of benzene in the bone marrow is up to 20 times higher than that in other tissues (3). In our experiments 25% of the animals in the MCA-initiated, benzene-treated group developed reticuloses and malignant lymphomas (Table 1). About the same frequencies were observed in the group receiving cantharidin without MCA initiation and in both asiaticoside groups. As reticuloses also occur spontaneously with increasing age of our hairless mice, it does not appear that benzene has any strong influence on the reticuloendothelial system in this connection.

General. The frequency and distribution of internal tumors other than reticuloses were constant in the different treated groups and were well comparable with the results from study of other, untreated hairless mice by Deringer (6). However, several questions arise concerning possible causes of the reticuloses observed. Was the increase of these lesions after cantharidin painting a specific promoting effect, or was it an unspecified toxic effect, acting by lowering the resistance of the animals to latent reticuloses?

The development of reticuloses (reticulum cell neoplasms) has been studied by many workers and is reviewed by Dunn (8, 9). These tumors have been induced by various carcinogens and also by benzene injections, but they may also have a very high spontaneous frequency. The frequency increases after thymectomy, and a viral etiology has also been suggested. The yield of reticuloses has been found to increase after various skin treatments, such as ether (9) and 3,4-benzpyrene painting (7), although Chouroulinkov (I. Chouroulinkov, personal communication) did not find increased frequency after long-term treatment with carcinogenic substances.

In this study the mice receiving different kinds of treatment were chosen randomly from a group of identical mice. Thereafter they were studied for 2 years under conditions which, apart from the painting, were identical. It is obvious that, when mice are kept alive so long and the tumor yield is very late, many unspecific factors may modify the tumor formation. Although the differently painted groups of mice may be directly compared, it is therefore possible that the frequency of the same tumors in these mice may be different under other environmental conditions or during another year.

Finally, the question arises as to what extent the tetrazolium method can predict carcinogenicity. One single application of cantharidin or asiaticoside gave values of formazan production from tetrazolium that indicated weak carcinogenic potencies. However, the development of skin tumors required repeated applications during a very long time period. During this period cantharidin even temporarily inhibited the development of papillomas after MCA initiation. However, the mitochondrial damage, which is probably the basis for the altered pattern of formazan production after carcinogen application, may be a specific toxic "side effect" of the substances.

In any case, the classical promoter, croton oil, which also is a complete carcinogen in the skin (2) shows the same reaction pattern with the tetrazolium method as cantharidin and asiaticoside (13).

We conclude that the inhibiting effect of cantharidin upon the development of papillomas in previously initiated skin is not in disagreement with the fact that it is a weak carcinogen.

ACKNOWLEDGMENTS

The typing of the reticuloses were done in collaboration with Dr. I Chouroulinkov and Dr. M. Guérin, Laboratoire de Médecine Experimentale, Institut de Recherches Scientifiques sur le Cancer du Centre National de Recherche Scientifique, Villejuif, France, to whom we owe our sincere thanks. We also thank Mrs. Aasa Schjolberg for skilful technical assistance.

REFERENCES


Fig. 1. Reticuloendotheliosis of angiomatos type in the liver. The neoplasm forms cyst-like structures with papillomatous ingrowth into the lumen. The liver sinusoids are also infiltrated. H & E, X 640.

Fig. 2. Reticulohistiocytosis in the spleen. The follicular lymphoid structure is replaced by infiltrating histiocytic cells. H & E, X 640.

Fig. 3. Reticulolymphogranulomatosis (reticular cell neoplasm type B) in the liver. The sinusoids are grossly infiltrated by lymphocytes, granulocytes, reticular cells, and giant cells. H & E, X 640.

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Announcements

ANNUAL MEETING OF THE SOCIETY OF TOXICOLOGY

The annual scientific meeting of the Society of Toxicology will be held at the Waldorf-Astoria Hotel in New York City, New York, on March 18 to 22, 1973. Anyone interested may attend. Papers for the meeting may be submitted by members of the Society. Persons not members of the Society of Toxicology may also present papers at this meeting if the paper is sponsored by a member. Titles should be submitted to Dr. Joseph F. Borzelleca, Medical College of Virginia, Richmond, Virginia 23219, no later than October 2, 1972.

Program, accommodations, and registration information for the meeting will be sent to all members of the Society and to those nonmembers presenting papers. All others should contact Dr. Robert A. Scala, Secretary, Society of Toxicology, Esso Research and Engineering Co., P. O. Box 45, Linden, New Jersey 07036.

Errata

In the article, entitled “Reticuloses and Epidermal Tumors in Hairless Mice after Topical Skin Applications of Cantharidin and Asiaticoside,” by Ole Didrik Laerum and Olav Hilmar Iversen, which appeared in the July 1972 issue of CANCER RESEARCH, the following corrections should be made: on page 1463, the first paragraph under “Summary,” Line 5, this sentence should read, “A short-term tetrazolium test indicated that both compounds in association with benzene were weak carcinogens.”; on page 1463, the second paragraph in the “Introduction” section, Line 1, the word “Madécassol” should be deleted; on page 1466, the third paragraph under “Asiaticoside,” Line 2, this sentence should read, “To our knowledge carcinogenesis studies have never been performed with asiaticoside in benzene.”; on page 1467, the fourth paragraph under “General,” Line 3, this sentence should read, “One single application of cantharidin or asiaticoside in benzene gave values of formazan production from tetrazolium that indicated weak carcinogenic potencies.” Also, the legends to Figs. 1 and 3 were incorrectly transposed.

In the article entitled “Central Inhibition of Cellular Immunity to Leukemia L1210 by Isoantibody,” by Malcolm S. Mitchell, which appeared in the April 1972 issue of CANCER RESEARCH, the words “afferent” in Lines 8 and 11 of Paragraph 2 of the “Discussion” section on page 829 should have been “efferent.”
Reticuloses and Epidermal Tumors in Hairless Mice after Topical Skin Applications of Cantharidin and Asiaticoside

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