The Isolation, Characterization, and Antigenicity of Malignolipin-like Material from Normal and Tumor Tissue

H. G. PETERING1, G. J. VanGIESSEN, H. H. BUSKIRK, J. A. CRIM, J. S. EVANS, AND E. A. MUSSER

Biochemical Research Division, The Upjohn Company, Kalamazoo, Michigan

Summary

A malignolipin-like material has been identified in the tissue and blood of both normal and tumor-bearing individuals of several species of animals and of man. The material isolated by us, which had chromatographic characteristics identical with those defining malignolipin (Kosaki), was found to be heterogeneous and to consist of lipid loosely associated with 3 amines, 1 of which was identified as L(+)-glutamic acid. The purified amines did not contain phosphorus. Pierates of the heterogeneous moiety or of the 3 amines contained large amounts of sodium and potassium picrates. The antigenicity of our fractions was studied. Preliminary examination of the blood of cancerous and normal human beings for malignolipin-like amines has been made.

Introduction

As part of a series of studies on porphyrins, Kosaki and Saka (19, 20) reported that cancer tissue but not normal tissue could be stained with protoporphyrin III. This work formed the basis for the later postulation that cancer cells contained a unique phospholipid which was called malignolipin, and which was believed to be responsible for this staining difference. Kosaki and his associates isolated and characterized this substance and reported that it was composed of choline, spermine, phosphoric acid, and a fatty acid (11). Malignolipin was further characterized by an RF value of 0.38 using ascending chromatography in methanol, air dried, and then dried at 120°C for 24 hr. This last drying step, which results in a final moisture content of approximately 5% (4), is essential for good separation. Columns were washed 4 times with absolute ethanol, homogenized, refluxed for 30 min, filtered, cooled to 0°C, filtered again, and the filtrate evaporated to dryness. The residue was reextracted with hot ethanol, cooled, filtered, concentrated, and precipitated with 2 volumes of acetone. The acetone precipitate after several acetone washings was dried in a desiccator. The dry solid, designated Me, was then redissolved either in chloroform for further purification by column chromatography, or in petroleum ether for further solvent purification and eventual picrate formation (15, 18).

DEFINITION OF TERMS. Table 1 summarizes and defines the various fractions and the abbreviations that are used in this report.

EXTRACTION PROCEDURE. These procedures have been described by Kosaki et al. (15, 18) in great detail and will only be outlined briefly to define certain steps for later discussion. Freshly excised tissue (tumor or normal) was immediately put into 9 parts (w/v) of absolute ethanol, homogenized, refluxed for 30 min, filtered, cooled to 0°C, filtered again, and the filtrate evaporated to dryness. The residue was reextracted with hot ethanol, cooled, filtered, concentrated, and precipitated with 2 volumes of acetone. The acetone precipitate after several acetone washings was dried in a desiccator. The dry solid, designated Me, was then redissolved either in chloroform for further purification by column chromatography, or in petroleum ether for further solvent purification and eventual picrate formation (15, 18).

PURIFICATION BY COLUMN CHROMATOGRAPHY. A mixture of silicic acid and Celite (2:1) was washed 4 times with absolute methanol, air dried, and then dried at 120°C for 24 hr. This last drying step, which results in a final moisture content of approximately 5% (4), is essential for good separation. Columns were...
Petering, VanGiessen, Buskirk, Crim, Evans, and Musser

TABLE 1
SUMMARY AND DEFINITION OF TERMS USED

<table>
<thead>
<tr>
<th>Terms</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>M, M-complex, or maligino-lipin-like amine</td>
<td>An amine prepared as described by Kosaki et al. (10, 14) which reacts with ninhydrin and has an Rf value of 0.38*</td>
</tr>
<tr>
<td>Mc</td>
<td>An impure extract containing M</td>
</tr>
<tr>
<td>M1, M2, and M3</td>
<td>Three distinct amines into which M can be resolved</td>
</tr>
<tr>
<td>Mln</td>
<td>A lipid-free, acid-soluble fraction obtained from M1</td>
</tr>
<tr>
<td>S</td>
<td>An amine prepared as described for M but with an Rf value of 0.50-0.52*; said to be spermine by Kosaki et al. (10)</td>
</tr>
<tr>
<td>U</td>
<td>An amine prepared as described for M and S but with an Rf value of 0.50-0.58*</td>
</tr>
</tbody>
</table>

* By ascending chromatography in methyl alcohol:pyridine:water (6:7:1)

PAPER CHROMATOGRAPHY. Both ascending chromatography and a circular paper chromatographic method were used. Whatman No. 1 filter paper discs were used in the circular method, and Whatman No. 1 or Schleicher and Schuell 2043-A paper strips were used in the ascending method.4 Solvent systems used were Kosaki’s system of methyl alcohol:pyridine:water (6:7:1), 88% phenol-water (4:1), and isopropanol:HCl:water (4:1:1). Chromatograms developed in phenol were thoroughly washed in acetone before staining with ninhydrin and cupric nitrate (10); those developed in isopropanol:HCl were first sprayed with NH4OH to neutralize the HCl and thoroughly dried before staining.

Purification of Solvents. All solvents were purified by the methods described by Kosaki et al. (16) and met or exceeded its requirements.

Identification of M1 as Glutamic Acid. A column fraction rich in M1, obtained from the purification of Walker Mc, was carefully taken to dryness under reduced temperature and pressure, dissolved in 0.3 N HCl, and extracted several times with ether to remove the lipid material. The acid phase was decolorized with charcoal (Darco G-60), neutralized with 10 N NaOH, and lyophilized. The dry solid was extracted several times with boiling 95% ethanol and then with boiling methanol until a small amount of the extract spotted on a filter paper no longer gave a positive ninhydrin reaction. The extracts were combined and concentrated at reduced pressure (< 50°C) to a small volume to precipitate most of the NaCl present. The supernatant was combined with 2 volumes of acetone. The white precipitate (358 mg, 34% NaCl) was dissolved in 20 ml of methanol plus several drops of water. To this was added 1 gm of picric acid dissolved in 20 ml of 95% ethanol. Since no precipitate formed, the solution was carefully taken to dryness using benzene to remove the water. The dry residue was extracted with benzene and acetone to remove the excess picric acid. The insoluble fraction (180 mg) was extracted 3 times with 4 ml of methanol to remove the amine picrates present. The white insoluble material (70 mg) was shown by paper chromatography to be very pure M1.

The infrared spectrum of this material, run as a mineral oil mull, was identical with a standard spectrum for L-glutamic acid. The $\alpha_{19}$ in 6 N HCl was found to be +18.3°.

Preparation of Antisera to M-containing Fractions. Rabbit antisera to the M-containing fractions (Mc, M1, M2, etc.) or to extracts of tissue or blood were prepared by the method of Witebsky and Rose (29). The dried fraction to be used was dissolved in 0.9% sodium chloride solution at a concentration of 2 mg/ml. An equal volume of Freund’s incomplete adjuvant (5) was mixed with the saline solution. Dried, killed Mycobacterium smegmatis was then added to give a concentration of 2 mg/ml of final volume. Fresh solutions of the M-fractions were used for each immunization procedure.

The rabbits were injected twice weekly for 4 weeks; the 1st injection of 1 ml/rabbit was divided into 4 equal parts and each injected into a paw. Subsequent injections of 6 ml/rabbit were given s.c. into several sites on each animal. The final injection was given i.m. Trial bleedings from an ear vein were made early in the immunization schedule, and final bleeding was by heart puncture 3–10 days after the last injection. A minimum of 4 rabbits were used for each preparation. The pooled antisera were held frozen (−60°C).

Preparation of Antisera to Walker 256 Carcinosarcoma Cells. Walker 256 tumor (W-256) cell suspensions were obtained by the method described by Petering et al. (24). Washed cells (free of blood cells) were suspended in phosphate-buffered saline (PBS, pH 7.4) containing 5% Freund’s complete adjuvant. From 2 to 4 ml of the suspension containing 15–50 million cells were injected twice weekly for 4 weeks. The first 2 injections were made s.c. into multiple sites, the next 4 i.v., and the last 2 i.p. The rabbits were bled by ear after the 1st injection, again after the 6th and the final by heart puncture 3–10 days after the last injection. A minimum of 4 rabbits were used for each preparation. The pooled antisera were held frozen (−60°C).

Preparation of Rabbit Antisera to Rat Spleen Cells. The spleens from several Sprague-Dawley rats were placed in a sterile Petri dish, minced with a scissors under aseptic conditions, and transferred to a 50-ml Pyrex centrifuge tube with 10–15 ml of PBS containing antibiotics. The tissue was broken up into a fine suspension by manipulation with a nylon brush according to the method of Brocklehurst (3). The fine suspension was centrifuged

Downloaded from cancerres.aacrjournals.org on June 9, 2017. © 1967 American Association for Cancer Research.
and the cells washed several times with saline. The final saline suspensions were adjusted to a final cell concentration of 5-10 million cells/ml and used to immunize rabbits in the manner described for immunization with W-256 cells.

**ADSORPTION OF ANTISERA.** In order to determine cross-reactivity, the different antisera were adsorbed with the antigens under test in the following manner, the antigens being M-fraction in various stages of purification. The M-fractions were mixed with an alcoholic solution of lecithin and cholesterol in the ratio of 1:9:20 so that intimate association occurred, and the solvent was removed by evaporation. The dry residue was mixed with 0.8 ml of the antiserum, incubated at 37°C for 30 min, refrigerated for several hr, and centrifuged at 8,000 to 10,000 rpm at 0°C to recover the antiserum which was tested for activity.

**PREPARATION OF M-CONTAINING ANTIGENS.** Preliminary studies showed that the haptens of the M-fractions had to be combined with lecithin and cholesterol in order to demonstrate complement fixing activity. The ratio of the M-fraction:lecithin:cholesterol found to be the most satisfactory was 1:9:20 which was the same ratio used by Rapport et al. (25) for cytolipin II and by Maltaner et al. (22) for cardiolipin.

When dry, purified antigen was used, the lecithin and cholesterol were added by weight and the mixture dissolved in ethanol. After thorough mixing, the solvent was removed in vacuo and the residue suspended in 0.9% saline containing 1% MgCl$_2$. When the antigens were prepared from either blood or tissue the following method was used. One ml of unheparinized blood or 1 gm of minced tissue was added to 9 ml of absolute ethanol, refluxed for 30 min, cooled to 0°C, and filtered through Whatman No. 2 paper. The precipitate was washed with a small volume of ethanol, and the combined filtrate and wash mixed with 0.25 mg of lecithin and 5.0 mg of cholesterol. The solvent was removed by evaporation in vacuo, and the residue suspended in sufficient saline containing 1% MgCl$_2$ so that 1.0 ml contained the extract from 1.0 ml of blood or 1 gm of tissue.

The complement fixing activity of the above antigens was determined using a series of 2-fold serial dilutions. The dilution of the antigen varied according to its source while the dilution of the antiserum was based on the titration of the individual or pooled samples used. All tests on antigens extracted from blood or tissue were run blind. Complement was titrated daily and diluted to a concentration of 20 units/ml.

To each tube in the series of antigen dilutions were added 0.1 ml of the appropriately diluted antiserum (usually 1:40) and 0.1 ml of complement. This mixture was incubated at 37°C for 60 min, and 0.2 ml of 1% sensitized sheep RBC added and again incubated at 37°C for an additional 30 min. The endpoint was the lowest dilution not showing hemolysis. A photometric method was used to determine lysis. Controls were run using 0.1 ml of saline in place of the antigen, 0.1 ml of saline in place of the antiserum, 0.2 ml of saline in place of both antigen and antiserum, and 0.3 ml of saline alone.

**Results**

**ISOLATION OF MALIGNOLIPIN FROM TISSUE.** Mc was prepared from the various normal and tumor tissues listed in Table 2. Paper chromatography, using Kosoaki's method (10, 14), showed that all of the tissues listed contained an amine similar to malignolipin with $R_F$ 0.38, which was designated "M," and an amine with $R_F$ 0.50-0.52 which Kosaki et al. (10) called spermine but which we designated "S." In addition most of the tissues contained an amine at $R_F$ 0.56-0.58 which we designated "U" (unknown). Kosaki and coworkers never reported this amine, but it was studied further because of its frequent occurrence. Although the yield of Mc varied among different types of tissue, average yields from all normal and all tumor tissues were similar. In addition we have also found M to occur in normal rabbit liver, human mammary cancer tissue, and in the noncancerous portion of the same human mammary gland.

Picrotates were made from all of the tissues listed in Table 2. Paper chromatography showed that all of the above-mentioned amines were present in the unpurified picrotates. The picrotates were found to dissociate during chromatography, with picric acid migrating faster than the amines.

Highly purified M, S (Kosoaki's spermine), and U were obtained by chromatography over silicic acid-Celite columns using increasing concentrations of methanol in chloroform to elute the fractions. U was eluted first (40% methanol), followed in order by S and M (50% methanol). U and S emerged as fairly discrete peaks but M trailed out over a considerable number of tubes and gave no definite peaks. Each of these amines gave only a single spot in the Kosoaki's solvent system. Our yield of about 300 mg of M from 1000 gm of tissue was comparable to the yield of 15-73 mg/100 gm reported by Kosaki et al. (15) for various tumors. Yields from normal and tumor tissues were similar.

**CHARACTERIZATION OF MALIGNOLIPIN-LIKE AMINES.** Paper chromatography of M, S, and U in other solvent systems revealed

\[
\begin{array}{ccc}
\text{Type of tissue} & \text{Separate} & \text{Total yield} \\
& \text{batches} & \text{wet wt. (gm)} & \text{yield Mc} \\
& & & \text{Wt. (gm)} \\
\hline
\text{Tumor tissue} & & & \\
\text{Walker carcinosarcoma} & 15 & 44,450 & 136.0 \\
\text{(rat)} & & & 0.30 \\
\text{Ehrlich carcinoma (mouse)} & 6 & 10,353 & 20.6 \\
\text{Murphy-Sturm lympho
darcoma (rat)} & 1 & 1,508 & 3.3 \\
\text{Shay leukemia blood (rat)} & 1 & 730 & * \\
\hline
\text{Normal tissue (all rat)} & & & \\
\text{Liver} & 2 & 2,049 & 11.1 \text{Average } & 0.54 \\
\text{Intestines} & 2 & 1,062 & 1.8 \text{yield } & 0.16 \\
\text{Testicles} & 1 & 311 & 0.9 \text{yield } & 0.27 \\
\text{Kidney} & 1 & 344 & 0.8 \text{yield } & 0.23 \\
\text{Spleen} & 5 & 993 & 1.2 \text{yield } & 0.11 \\
\text{Lungs} & 4 & 1,088 & 1.9 \text{yield } & 0.17 \\
\hline
\text{Average } & & & \text{yield } & 0.30 \\
\end{array}
\]

* No weights were obtained since this preparation was used to prepare picrotates directly from the Me extract.
several additional amines as well as some lipoidal material. Purified M₁, a single entity in Kosaki's solvent, was resolved into 3 amines (designated M₁, M₂, and M₃) in the systems phenol: water (4:1) and isopropanol: HCl: water (4:1:1). Purified S and U ran as single spots in all 3 systems. Examination of column chromatography eluates using all 3 solvent systems showed that columns also resolved U, S, M₁, M₂, and M₃ in order, although there was considerable overlapping particularly of the M-fractions. Reexamination of Me extracts showed the presence of M₁, M₂, and M₃ in both tumor and normal tissue extracts, indicating that they were not artifacts produced by the columns.

Table 3 summarizes the chromatographic characteristics of these amines in 3 solvent systems using ascending chromatography. The R_F values for picric acid and glutamine are included since they interfere with the amine spots in some instances. Glutamine, in particular, interferes in the Kosaki system where its R_F value is identical with that of the M-amines. No evidence was found to indicate the presence of glutamine in any extracts which were prepared.

Because the amines were not completely separated on the columns, the small amounts of each obtained in a pure state made extensive chemical analysis difficult. Paper chromatography in the 3 solvent systems described above, revealed that the R_F value of M₁ was the same as glutamic acid while M₂ was the same as aspartic acid. The 3rd M-amine (M₂) could not be identified in this manner, although it was shown not to be glutamine, glutamic acid γ-ethyl ester, or β-hydroxyglutamic acid. Similarly, S was shown definitely not to be spermine since it did not have the same R_F values in the 3 systems. U also remained unidentified; however, data to be discussed later show that M₅, S, and U have other very similar properties.

M₁ was positively identified as L(+) -glutamic acid after extensive purification of a column fraction obtained from the purification of Walker 256 Me. The infrared spectrum of pure M₁ in mineral oil was identical with a reference sample of L-glutamic acid. The [α]_D was found to be +18.3° in 6 N HCl. Although this is less than the known value of 31.4°, it is probable that our preparation was partially racemized during the extensive purification procedure.

M₂ could be further separated into an amine and a lipid material by distribution between 0.3 N HCl and ether, conditions which Kosaki et al. (11, 15) claimed caused hydrolysis of malignolipin with the formation of choline and spermine. However, the acid-soluble amine obtained by this distribution and designated M₂a, showed the same chromatographic behavior as M₁ in all 3 solvent systems. Additional studies, however, revealed that M₅, and probably also M₁, M₃, S, and U, was dissociated during chromatography into an amine and a lipid fraction which ran separately. There was no evidence of any hydrolysis of M₂a, taking place during this separation procedure, since tests for choline, spermine, or any other primary amine in the acid phase were negative, and the R_F value of M₂a remained unchanged. The amine was also stable to heating in 4 N HCl for 3 hr at 100°C. Furthermore, attempts to prepare the picrate of M₁ yielded instead the picrate of M₂a with the separation of a lipoidal material into the mother liquor.

Preliminary investigation of the lipoidal moiety which was associated with M₂a to form M₂ was made using gas phase chromatography. Silicic acid column fractions from the purification of Walker 256 Me and normal rabbit liver Me were separated into ether- and acid-soluble portions. The ether-soluble portions were transesterified using perchloric acid and methanol, and subjected to gas phase chromatography. The ether-soluble fractions, obtained from Me-containing eluates of Walker 256 Me, contained long chain fatty acids of C₂₀, C₂₂, and C₂₄ length. Those fractions eluted earlier than Me, and all fractions from normal rabbit liver Me contained only the normal distribution of fatty acids of chain length C₁₈ or less. The C₁₈-C₂₄ fatty acids from Walker 256 Me were found to be saturated, which is contrary to Kosaki et al. (15, 17), who suggested that the highly unsaturated fatty acid present in malignolipin accounted for the unusually high picric acid content of their picrates.

Preparation and purification of amine picrates. Much of Kosaki's work, both chemical (15, 17, 18) and biologic (12, 13, 21), was based on the use of "pure malignolipin" originally isolated as malignolipin picrate. Since several amines were found which fulfilled Kosaki's criteria for malignolipin, an extensive investigation was made of the formation and purification of their picrates. Initially, picrates were prepared directly from Me using Kosaki's methods (15, 18). However, these proved extremely difficult to purify. They were generally mixtures that contained most of the amines listed in Table 3 and much lipid material; some were free of S and U, but most contained M₁ and M₂ and frequently M₃. Several picrates containing no ninhydrin-reacting amines were obtained, which is similar to the findings of Sax et al. (26). Repeated recrystallization removed much of the interfering lipid material but did not separate the various M-picrates. Replacing benzene with toluene containing 0.5% water as the medium for precipitation improved yields and purity but again did not separate the M-components. The lipid moiety also inter-
TABLE 4

**Physical Properties of Several Purified Picrates of Malignolipin-like Amines**

Picrates were prepared by several methods as described in the text. The % picric acid was determined spectrophotometrically at 360 nm in 95% ethanol. All melting points were determined on a Fisher-Johns hot stage and are uncorrected. Calculated values for picric acid and sodium and potassium picrates are included for comparison.

<table>
<thead>
<tr>
<th>No.</th>
<th>Amine</th>
<th>Source</th>
<th>Melting range (°C)</th>
<th>Picric acid (%)</th>
<th>Elemental analyses (%)</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>K</th>
<th>Na</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>S</td>
<td>Tumor</td>
<td>267°-273°</td>
<td>82.6</td>
<td></td>
<td>27.2</td>
<td>2.2</td>
<td>15.1</td>
<td>0.3</td>
<td>7.6</td>
</tr>
<tr>
<td>II</td>
<td>U</td>
<td>Tumor</td>
<td>255°-260°</td>
<td>74.7</td>
<td></td>
<td>26.3</td>
<td>1.8</td>
<td>15.0</td>
<td>12.1</td>
<td>1.1</td>
</tr>
<tr>
<td>III</td>
<td>M₁</td>
<td>Tumor</td>
<td>270°-275°</td>
<td>86.2</td>
<td></td>
<td>27.3</td>
<td>2.0</td>
<td>15.0</td>
<td>0.3</td>
<td>6.0</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td></td>
<td>270°-275°</td>
<td>83.1</td>
<td></td>
<td>28.0</td>
<td>1.6</td>
<td>16.3</td>
<td>0.3</td>
<td>6.0</td>
</tr>
<tr>
<td>V</td>
<td></td>
<td></td>
<td>&gt;300°</td>
<td>74.6</td>
<td></td>
<td>23.5</td>
<td>1.7</td>
<td>13.6</td>
<td>11.7</td>
<td>5.3</td>
</tr>
<tr>
<td>VI</td>
<td></td>
<td>Normal rat spleen</td>
<td>240°-248°</td>
<td>63.5</td>
<td></td>
<td>25.4</td>
<td>1.9</td>
<td>13.9</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>VII</td>
<td>M₂</td>
<td>Normal rat spleen</td>
<td>180°-200°</td>
<td>63.5</td>
<td></td>
<td>27.5</td>
<td>2.1</td>
<td>12.5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>VIII</td>
<td>M₂</td>
<td></td>
<td>Picric acid (calc.)</td>
<td>121°-122°</td>
<td>100.0</td>
<td>31.5</td>
<td>1.3</td>
<td>18.3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sodium picrate (calc.)</td>
<td></td>
<td>90.8</td>
<td>28.7</td>
<td>0.8</td>
<td>16.7</td>
<td>9.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Potassium picrate (calc.)</td>
<td></td>
<td>85.4</td>
<td>27.0</td>
<td>0.8</td>
<td>15.7</td>
<td>14.6</td>
<td></td>
</tr>
</tbody>
</table>

* For abbreviations, see Table 1.

* Phosphorus analysis was negative for all samples; oxygen could not be determined because samples exploded.

* Did not contain any amine which reacted with ninhydrin.

* Insufficient sample for complete analysis.

Our best preparations were obtained after removal of the lipid components by distribution between 0.3 and 1.0 x HCl and ether, followed by preparation of the picrate in ethanol. Any picrates precipitating from ethanol were removed, the ethanol concentrated to a small volume under vacuum, and the remaining picrates were precipitated with 10 volumes of toluene. Further purification could be made by recrystallization from ethanol: toluene, dioxane:toluene, or acetone:toluene. This procedure was used to purify picrates from either Mc extracts or column fractions, although the Mc-picrates required many recrystallizations to obtain good material.

A number of reasonably pure picrates were obtained from both normal and tumor tissues by the various methods or combinations of the methods outlined above. The physical properties of several are presented in Table 4. These compounds were obtained in crystalline form and were shown by paper chromatography to contain picric acid and only a single amine or no amine. Tests on these crystalline picrates for aromatic amines, unsaturated bonds, aldehydes, carbohydrates, alkaloids, and sugars were negative.

Regardless of the source of the picrate or the presence of a primary amine, the physical properties of these compounds were all similar to each other but different from picric acid. Their melting points were also much higher than the 123°C reported by Kosaki et al. (15), but agree with the melting point of 258°-265°C reported by Kamat (9) and the several melting points reported by Sax et al. (26). The low melting points and picric acid contents of Compounds VII and VIII (Table 4) may be due to the presence of unremoved lipid. The picrates contained no phosphorus, which agrees with both Kamat and Sax et al. but not with Kosaki et al. (11). The picric acid contents, determined spectrophotometrically in 95% ethanol using the 360-nm absorbance of picric acid, agrees quite well with Kosaki et al. (15) and Sax et al. (26), who used other methods. Our values for C, H, and N are similar to those reported by Sax et al. (26); Kamat reported similar values for C and H but a much higher value for N (22.5%).

All samples of picrate contained significant amounts of sodium and potassium, the total of both varying from 6.2 to 17.0%. These elements, however, occurred in no particular ratio as Sax et al. suggested, nor were the amounts in normal or tumor tissue picrates different. The picric acid, sodium, and potassium content totalled approximately 90%; thus the amine or other materials represented only 10% of any of these compounds.

Infrared analyses of our picrates were inconclusive. Kosaki et al. (17) reported the spectrum for malignolipin picrate in acetone solution, which requires relatively large amounts of material. Only Compounds I, II, and III (Table 4) were available in sufficient quantities to use this method. The spectra of I (S) and III (M₂) definitely resembled those of Kosaki et al. However, due to the lack of fingerprints and the large amount of picrate ion present, a positive identification could not be made. Spectra taken in Nujol mulls also had little value; however they showed that Compound VI was primarily an inorganic picrate, probably...
Malignolipin Blood Tests for the Diagnosis of Cancer. Kosaki and his associates have proposed 2 methods for detecting malignolipin in blood as a means of diagnosing cancer. One method (10, 14), essentially that used to extract malignolipin from tissue, utilized paper chromatography to determine the presence of malignolipin in the final extract. The 2nd method (18) involved a similar extraction followed by preparation of malignolipin picrate and several purification steps. A positive test gave a yellow picrate in the final step. We have investigated both methods, using extreme care to follow the original procedures.

Using the 1st method, we found no consistent differences in the M-amines between normal and early or late cancerous blood from rats, mice, and dogs. Continuing this investigation, we again failed to find any significant differences between 8 normal, non-cancerous human blood samples and 9 samples from human cancer patients. We consistently found the amines described earlier in all of the blood samples regardless of source or type.

The same group of human tumor bloods and 4 other noncancerous human bloods were also tested using the paper chromatography method which Kosaki et al. claimed was subject to fewer possible errors. All normal bloods gave a negative result but only 3 of the 9 tumor bloods gave a positive response. Duplicate samples of 1 tumor blood gave negative results both times. All tumor bloods came from patients with late, extensive, metastatic cancer of various types.

Antigenic Activity of Malignolipin. Kosaki et al. (12) reported that the injection of the ammonium salt of malignolipin into Wistar rats produced antibody to malignolipin, which could be removed by adsorption with tumor cells, but not with normal erythrocytes. These investigators obtained complement fixation for the binding of complement by cytolipin H (25) and cardiolipin (22), enabled us to detect the complement-fixing activity of M2. Rabbit antisera prepared from either the lipid-amine complex (M2) or the acid-soluble amine (M3) bound complement in the presence of ethanol-extractable antigens from normal as well as tumor tissues from several species. Witebsky in 1929 (28) made similar observations using antisera prepared to Sarcoma 180.

The chemical nature of the antigen (or hapten) present in either normal or neoplastic tissues which reacts with antisera to either M2 or M3 remains unknown. We have found that the material that combined with antisera to either M2 or M3 is not associated with mono-, di-, or triglycerides, cholesterol esters, sphingomyelin, or the cerebrosides fractions. We do not feel that the antigen(s) is the same as cytolipin H (25), the alcohol-soluble protein of Seibert et al. (27) or oncolipin (1) on the basis of chemical and serologic tests.

Rabbit antisera to M3 were also studied for their action on living Walker 256 tumor cells by a modification of the method of Arai and Suzuki (2), in which viable tumor cells reduce methylene blue. The degree of dye reduction indicates the number of viable cells left after a given incubation period with a cytotoxic agent. Rabbit antisera to M2, in dilutions up to 1:640 plus complement (guinea pig serum), caused destruction of tumor cells which was comparable to that caused by similar dilutions of rabbit antisera to Walker 256 cells and to normal rat spleen cells. These antisera also prevented "takes" when inoculated with Walker 256 tumor cells before being implanted into rats; dilutions 1:40 allowed 20% takes while 1:4 dilutions prevented all takes. Controls of Walker 256 cells plus guinea pig serum showed 100% takes. These results indicated that an antibody capable of destroying tumor cells may be produced from antigens found in both normal and tumor tissues.

M-fractions collected during the silicic acid column purification of Walker 256 M2, which showed C'-fixing activity, were evaluated for antigenic activity by their capacity to neutralize M2-antiserum cytotoxic activity in the Arai-Suzuki test system. By this method, it was found that the major portion of the antigen from the columns was uniformly distributed in all of the collection tubes obtained prior to the elution of M2, although the M2 fraction also contained neutralizing antigen. Thus the elution pattern of the antigen did not follow that of any of the M-amines—namely, M1, M2, or M3—all of which show RPs identical with that reported by Kosaki (0.38) in his system.

Furthermore, it was found that antisera to M1 and to Walker 256 cells were neutralized by the antigen in M-fractions, but the antiserum to rat spleen cells was not. Thus there is cross-reactivity between M-fractions (especially M2) and the antigens to Walker 256, but no cross-reactivity between M-fractions and rat spleen cells.

These results, although not exhaustive and not completely definitive, do suggest that the amines which we isolated and which conform to Kosaki's chromatographic definition of malignolipin are not responsible in themselves for the antigenicity found in the various fractions.

Discussion

Our purpose in beginning these studies was to determine if malignolipin was indeed a new phospholipid unique to cancer tissue, and if its presence in blood could be used to diagnose cancer. The data presented here show that the heterogeneous substance (M) which conforms to Kosaki's original chromatographic definition of malignolipin is not a phospholipid, that it is not unique to cancer tissue, and that the blood tests for its presence probably are not suitable for the detection of cancer.

M, a malignolipin-like substance isolated according to the method of Kosaki et al. (10, 14) is actually composed of 3 closely related simple amines and amino acids which are loosely complexed with a lipid moiety. Under the conditions of isolation (according to Kosaki et al.) this mixture of amines was not resolved into its components and therefore appeared to be a single amine. When Kosaki et al. attempted to use other methods of purification, particularly column chromatography, they experienced an apparent loss of malignolipin (16).

Our data suggest that they probably separated these amines with no real loss of malignolipin.
Two of the amines of the M-complex have been identified as L(+)-glutamic acid and probably aspartic acid. The 3rd amine in the M-complex (M2) and the 2 other amines consistently found in these preparations (S and U) remain unidentified. Although the fraction S has some of the characteristics attributed to spermine by Kosaki et al. (10), it is not spermine, and it is improbable that the authors were dealing with spermine since their identification was based solely on an Rf value in a single solvent system. Our analytic data suggests that M2, S, and U are small-molecular-weight amines which contain no phosphorus or carboxyl groups. By paper chromatography we have investigated numerous amines and amino acids which might fit these requirements without finding any clues to their identity.

Our picrate studies support the above conclusions and also show that isolation and purification by means of picrates is difficult. The large amounts of sodium and potassium picrates formed, along with the other picrate ion present in the various preparations, accounts for up to 90% of the picrate obtained. The remaining 10% contains the amines and any impurities. This has presented obvious difficulties in purification and analysis.

The presence in the extracts of a lipid material is somewhat puzzling. The presence of the C16-18 fatty acids in the M-amine fraction from tumor tissue but only normal length fatty acids in the other fractions of tumor tissue and in all amine fractions (including M-amines) from normal tissue is a provocative finding which requires further study. Any free fats and fatty acids should have been eluted under our conditions prior to the amines. This suggests that the lipid somehow complexes with the amine(s), yet the binding must be very loose since the lipid can be removed using 0.3 N HCl and ether. No specific antigenic activity was found to be associated with the lipid material or the amine complex.

We have not confirmed the validity of either of the blood tests proposed by Kosaki et al. for the detection of cancer. Our finding that malignolipin is probably not a phospholipid but several simple amines casts considerable doubt on the chromatographic method. In addition, the picrate method may not actually measure malignolipin picrate as claimed by Kosaki et al. but some other presently unidentified picrate, since picric acid can complex with many compounds other than amines. Nevertheless, in its present form, this method is still unsuitable as a test for cancer.

Attempts to devise a diagnostic test for cancer utilizing antisera to M2 or M3, was unsuccessful. Most of the column fractions from the purification of either tumor or normal tissue neutralized antisera to M3, which is both C'-fixing and cytotoxic for Walker 256 cells in the presence of C'.

Acknowledgments

We gratefully acknowledge the assistance of G. D. Mengel, J. E. Rogers, E. E. Smith, P. Dickson Edwards, M. Kogiku, W. Schroeder, and E. B. Nadolski in carrying out these studies.

References


The Isolation, Characterization, and Antigenicity of Malignolipin-like Material from Normal and Tumor Tissue


| Updated version | Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/27/1/7 |

| E-mail alerts | Sign up to receive free email-alerts related to this article or journal. |
| Reprints and Subscriptions | To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org. |
| Permissions | To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org. |