EFFECT OF POLYSACCHARIDE ON THE YOSHIDA SARCOMA CELLS (Preliminary Report)
(With Plates XXIII and XXIV)

YOSHIHIRO HAMASHIMA, HIDEO KANAMORI, and YOSHIHARU KUNIEDA
(From the 1st Division of Pathological Institute, Faculty of Medicine, Kyoto University. Director: Prof. K. SUZUE, M.D.)

In an extensive study on the type-specific polysaccharide of the Capsular Pneumococcus, Heidelberger, Kendall, Avery, Mc Leod and others had elucidated the fact that the bacterial polysaccharide served as the hapten immunologically, and studied further the possibility of applying this knowledge to clinical therapy. Recently, the study of polysaccharide is being made from various angles. Matsu-bara et al contended, as the result of their serial studies on fractional components of the neoplastic tissue, that their method was helpful in the early diagnosis of cancer and pregnancy. Recent success of E. J. Hehre in synthesizing polysaccharides promotes the further expansion of our knowledge in this field. While undertaking a study on the specific activity of the bacterial polysaccharide on single cell, the authors examined the specimens of polysaccharide prepared from sarcoma tissue for their effect on the growth of the sarcoma. This study has been motivated by an expectation that the tumor-inhibitory effect of such specimens, if there may be any, might be tumor-specific, and there may be less side-actions in this case than in the case of using other agents.

As the source of polysaccharide, Yoshida sarcoma (ascites sarcoma of the rats) was used.

EXPERIMENTAL

1. Preparation of polysaccharide

Yoshida sarcoma, as growing subcutaneously, was pooled, minced and homogenized. The homogenate was added to 5 volumes of distilled water, and was allowed to stand overnight at 0°C. The supernatant liquid after centrifugation was removed, the precipitate was washed with water repeatedly, and the washings were added to the supernate. Every 100 cc of the combined liquid was mixed with 10 g CH₃COONa·3H₂O and 1 cc CH₃COOH, and shaken vigorously after addition of 1 to 1.5 volume of 95% ethyl alcohol. After standing overnight in the refrigerator, the supernatant liquid was removed by means of siphon, and the residue was centrifuged. The precipitate after centrifugation was dissolved in 50 cc distilled water, 5 g CH₃COONa·3H₂O and 0.5 cc CH₃COOH added, and shaken with a mixture of 10 cc CHCl₃ and 1.6 cc
buthyl alcohol (normal) \(\text{CH}_3(\text{CH}_2)_2\text{CH}_2\text{OH}\) for 1 to 2 hours. Centrifugation at 2000 r.p.m. for 30 minutes to 1 hour resulted in the separation of the liquid into chloroform and n-buthyl alcohol and shaken. This procedure was repeated until the aqueous fraction became negative to Biuret reaction, whereupon it was precipitated by 95% ethyl alcohol. The chloroform fraction was pooled, centrifuged repeatedly to eliminate the aqueous component. The above precipitate dissolved in 20 cc distilled water, was added to 1 to 1.5 volume of alcohol. White mass of precipitate forming in this mixture was removed by centrifugation at 2000 r.p.m. for 15 minutes. When the supernate was mixed with 4 g \(\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}\) and 0.4 cc \(\text{CH}_3\text{COOH}\), polysaccharide formed.

Specimens of polysaccharide were examined then for the presence of starch or glycogen by iodine reaction, and for phosphate radicals by ammonium molybdate test. When these tests were positive, specimens were dissolved in distilled water and precipitation was repeated with acetic acid. When negative, addition of re-distilled alcohol gave a pure precipitate of polysaccharide. The precipitate was washed again with alcohol, and dried in vacuum with \(\text{CaCl}_2\) and then with \(\text{P}_2\text{O}_5\).

Polysaccharide was obtained as white powder, water soluble and alcohol-insoluble.

Yield:

<table>
<thead>
<tr>
<th>Sarcorna</th>
<th>Polysaccharide obtained</th>
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<tbody>
<tr>
<td>32 g</td>
<td>23 mg</td>
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<tr>
<td>45 g</td>
<td>116 mg</td>
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<tr>
<td>36 g</td>
<td>90 mg</td>
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<tr>
<td>22 g</td>
<td>65 mg</td>
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2. Animal Experiment

Four to five days after rats were given the intraperitoneal inoculation of Yoshida sarcoma, they were injected with polysaccharide intraperitoneally in dose of either 15 mg, 40 mg or 60 mg in the form of saline solution. Specimens of peritoneal fluid were withdrawn 30 minutes, 1 hour, 3 hours, 6 hours, 12 hours, 24 hours and 36 hours after the injection, and were examined for the changes of cells, whereby the tumor-inhibitory activity of polysaccharide was compared with that of nitrogen mustard.

3. Effect of Polysaccharide

Administration of polysaccharide was nearly innoxious to animals, as judged from the result of the intraperitoneal injection, and the manifestation of the direct effect on tumor cells took place only slowly. However, when a fairly large dose was administered, the cytoplasm of cells, both in mitosis and at-rest, were subject to specific alteration. Namely, immediately following the leukocytic response 3 to 6 hours after the injection, the maximal inhibition occurred. The number of tumor cells decreased gradually thereafter, becoming sparse after 12 hours. However,
the inhibitory effect expired at the latest 14 to 15 hours later, and gave way to the resumption of tumor cell proliferation, in case no repeated injections were instituted.

The cytoplasm was the only part involved in the pronounced morphological changes such as disappearance, plasmolysis, edema, swelling and deformity, present occasionally associated with maximal inhibitory activities. These findings are highly suggestive of a specific reaction occurring between polysaccharide and the organic constituents of the cytoplasm of tumor cells, although there is no information as to what the underlying mechanism will be. That a minority of tumor cells remained free from any alteration, and that the resumption of tumor cell proliferation took place at the time when inhibitory activity expired, suggest the fact that polysaccharide is of no effect on a certain phase of the cell development (probably the most active, young cells or such cells as holding a particular constitution of the ingredients where the cells are not susceptible to the action of polysaccharide).

Administration of Polysaccharide in dose of 15 mg.

30 minutes after injection, there was as yet no manifestation of the inhibitory activity, although leucocytotic reaction was most active in this stage, degenerated monocytes being noticed occasionally. As to the picture of cell division, the chromosomes were observed distinctly, their number ranging between 30 and 40. Relative to the leucocytes, number of the neutrophils increased steadily, reached maximum nearly at the 3rd hour, but began to fall around the 16th hour with concomitant cellular degeneration. Tumor cells, as stained supravitally, were of usual size, the nucleoli being large and stained distinctly, mitochondria in usual number and azure granules in normal distribution in the paranuclear area. One hour after injection, the cells showed a picture of slight nuclear deformity, atrophy and thickening of nucleoli, although pronounced swelling of cytoplasm was encountered occasionally, being several times of the usual size. The alteration of the paranuclear area of cytoplasm, particularly where there was rich distribution of azure granules, was outstanding in that it was initiated by small vacuole formation. It seemed that dysfunction occurred first in Golgi apparatus. Cells appeared generally hypertrophic, and showed an increased basophilia, with nuclei nearly compact. Along with the steady increase of leucocytic reaction, migration of macrophages occurred, but it subsided by the 24th hour to restore tumor-cells to the previous state of active proliferation. The lesions of the cytoplasm remained for as long as 6 hours, but the growth of tumor cells was resumed thereafter, the complete resumption being accomplished by the 24th hour.

Administration of Polysaccharide in dose of 40 mg.

Specimens of the ascites, in the state of pure culture of tumor cells prior to injection, showed the picture of leucocyte reaction 30 minutes to 1 hours after the injection, and the specimens were loaded with numerous leucocytes, two several times as many as tumor cells, where every one tumor cell was found surrounded
by a mass of leukocytes. Although no marked change was yet produced in tumor
cells in this stage, cytoplasm swelled up markedly after the 3rd to 6th hour and
looked hyaline-transparent, probably due to a decreased viscosity of the cytoplasm,
and this gave an impression as if plasm-membrane has been distended. Increased
number of tumor cells showed the picture of perinuclear vacuole formation. It seemed
that polysaccharide caused the tumor cells to be slowly degenerated centripetally
from the periphery. It was found in every instance that the cytoplasm was the
only part involved in the degeneration, and the nuclei and nucleoli remained intact,
and so did the chromosome in the metaphase and anaphase of the cell division.
The cytological manifestation of the tumor-inhibitory activity was similar between
polysaccharide (in the dose of 40 mg) and nitrogen mustard in many aspects, except
for the difference that the former affected the cytoplasm while the latter affected
the nucleus. Besides this, a marked difference arose in connection with how fast
the inhibitory effect came into existence; namely, the severity of the changes 30
minutes after the administration of nitrogen mustard was equivalent with that
resulting 3 to 4 hours after polysaccharide injection.

Administration of Polysaccharide in the dose of 60 mg.
The outstanding feature was the specific destruction of the resting cells, and the
cells in mitosis seemed to be free from any pronounced primary lesion. Namely,
the changes of resting cells were initiated with such patterns of lesion as plasmolysis,
hyaline swelling of cytoplasm, standing of nuclei out of softened cytoplasm. Along
with these changes, atrophy and destruction occurred in the leukocytes, which were
partaking in the already active leukocytosis. Six hours after injection, the leukocytic
reaction including neutrophilia subsided, the perinuclear vacuole formation as well
as lysis of cytoplasm being the outstanding picture. In case further destruction
proceeded, this picture was modified by the presence of naked nucleus similar to
the one caused by Nitromin or Colchichin administration. However, nuclei were
not involved whatever in the development of cytoplasmic degeneration. Some of
the cells with highly degenerated, softened cytoplasm were hardly stained by the
dyes and looked hyaline transparent, although they were seldom subject to such
type of degeneration as formation of droplet-sized vacuoles. These two types of
degenerated cells showed none of the solid structure such as neutrophil granules,
Golgi apparatus, mitochondria or metachondria.

**CONSIDERATION**

It has been demonstrated that the tumor inhibitory activity of polysaccharide was
limited within the scope of cytoplasm, and seldom involved nuclei. Polysaccharide
failed to meet the necessary condition for a cancer-inhibitory agent that it interferes
with the cell division and growth. The characteristic feature of the activity of
polysaccharide was that it induced an alteration specifically in the cytoplasm of
tumor cell. The fact that no preliminary knowledge was available as to the adequate dosage, together with the fact that only few animals were studied for the effect of successive administrations hindered the authors greatly from reaching more solid results. Since not only the nucleus-attacking agent, but also the cytoplasm-poison as well may serve as the anti-carcinoma agent, and since fairly marked lesion was actually produced in the cytoplasm of tumor cells in the above experiment, although slowly in its onset and transient in its duration, it may well be expected that someday we may give polysaccharide to tumor-bearing subjects in an improved manner with gratifying result. The synthesis of polysaccharide being possible today, it may be duly hoped that more powerful compound will be synthesized by substituting the active radicals with those of higher activity. It may be possible, too, to duplicate the effect of polysaccharide by covering the drawbacks of the present method by simultaneous administration of other drugs. It deserves attention that the polysaccharide elaborated by the tumor tissue seems to be injurious to the growth of the same tumor. There is a need of further investigation on this subject, particularly on the possibility of DNA synthesis in the sarcoma tissue, because, in case such synthesis occurs in the sarcoma tissue, effect of polysaccharide on DNA synthesis should be a subject of fruitful research.

CONCLUSIONS

(1) Specimens of polysaccharide, obtained by fractionation at low temperature from a mass of the subcutaneous growth of Yoshida sarcoma, act specifically on the cytoplasm of Yoshida ascites tumor cells. The earliest manifestation of the cytoplasmic lesion takes place in the paranuclear area.

(2) Average yield of polysaccharide is 1.104 mg per gram of the tumor tissue.

(3) Administration of polysaccharide is innoxious to the living body. As judged from the results of parallel experiments with different doses, varying as 15, 40 and 60 mg, tumor-inhibitory effect was proportionate to the dose administered, but was transient irrespective of the doses.

ACKNOWLEDGMENTS

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REFERENCES


EXPLANATION OF PLATE XXIII

1) Administration in dose of 15 mg.
Fig. 1, Fig. 2. It is obvious from both photographs that the initial vacuoles formation takes place at the paranuclear area. No abnormality occurs in the mitosis.
Fig. 3, Fig. 4. Polysaccharide attacks the most active part in metabolism and leads to the degeneration associated with vacuoles formation.

PLATE XXIV

2) Administration in dose of 40 mg.
Fig. 5. 30 minutes: Appearance of vacuoles.
Fig. 6. 1 hour: Perinuclear vacuole formation.
3) Administration in dose of 60 mg.
Fig. 7. 4 hours later: The picture indicates the lysis and disappearance of cytoplasm.
Fig. 8. 6 hours later: Diminished size of cytoplasm as compared with the size of the nucleus.
要 旨

多糖類の吉田肉腫細胞に及ぼす影響 (予報)

浜島義博・金森秀夫・元枝義治
(京都大学医学部病理学教室)

II 及び III Capsulare Pneumokokken の型特異性多糖類を抽出してその特異性免疫反応について研究中、その中と抗癌体反応に興味ある事実を認めた事よりヒントを得、この理を吉田肉腫細胞に応用してみたならば如何なる結果を得るだろうかと、肉腫中多糖類を低圧下に抽出しその影響を観察したところ若干の知見を得たので記述した。しかし現在なお、持続投与例並びに追加試験を総合中であり未結論のままであるが、得た所見を予報として記載した。肉腫多糖類は醗醗反応とクロロフォルム、プタノール抽出法を利用し、アルコール沈殿をもって得、反復抽出を施して精製品を得た。吉田肉腫細胞に及ぼす影響は、純培養状態にて直接癌陰内に食塩水にて溶解した多糖類を注入して腹水細胞に接解させた直接効果の有無を時間を追い検討した。肉腫細胞原形質に選択的に融解作用を及ぼし、この際、原形質のいわゆる明細部に最も早く障害を引き起こすことを知った。核に対する直接作用は全く認められなかったが、60 mg の大量投与の際には、原形質が完全に融解消失して裸核型を呈するに到る細胞をもあった。しかし腫瘍抑制効果としては作用は一時的で永久性に欠けるが、他の抑制物質（例えば Nitrogen Masturd や Colchichin）に比べて生体に及ぼす害の比較的少ない利点があり、その連続投与効果についても今後研究中である。