Arch Jap Chir 47 (2), 156~168, März, 1978

Studies on N-butyl-N-nitrosourethan-Induced Cancers of the Esophagus and the Forestomach of Rats

TADAMICHI YASUMOTO

The Second Surgical Division, Yamaguchi University School of Medicine (Director : Prof. Dr. Koichi ISHIGAMI)

Received for Publication Dec. 28, 1977

Introduction

Many authors have reported that Bleomycin (BLM) is remarkably effective on well-differentiated squamous cell carcinoma[3)10)13)18)20)]. BLM distributes to squamous epithelium, such as the skin and the esophagus, lungs and kidneys, following intravenous and intramuscular injections[4)5)7)15)25)26)]. In these tissues, the drug is inactivated more slowly[6)15)]. The author studied BLM distribution in the esophagus and squamous cell carcinoma of rats induced by N-butyl-N-nitrosourethan (BNUR) by means of the histochemical method. Moreover, the histological changes of the cancer treated with BLM were observed by the light and the polarizing microscopies.

(1) *Neoplasmas in rats that received BNUR administration.*

Materials and methods : In the first place, the neoplasmas of the esophagus and the forestomach of rats were produced by the administration of carcinogens. According to TAKEUCHI's report in 1974, experimental animals were Donryu rats weighing about 150 grams and the carcinogen was BNUR[21)]. Forty Donryu rats were administered with BNUR in drinking water at the concentration of 0.04% for 20 to 40 weeks. Twenty-nine rats which had survived for more than 20 weeks were used for the investigations.

Results : All of the 29 rats had multiple papillomas in the esophagus and the forestomach (Photo. 1). The longer the duration of administration of the drug was, the more the papillomas were induced. The occurrence of the papilloma had extended from the esophagus to the forestomach. Macroscopically, the papillomas were divided into two types, one was the polypoid type with a smooth surface and the other had a dendritic shape. The histology of the esophagus and the forestomach revealed sporadically hyperplasia of the epithelium and atypical proliferation of the basal layer. Among some of these papillomas, carcinomatous changes were observed in part. It is thought that some carcinomas may be induced from the papillomas. The carcinomas were induced in 26 of 29 rats and the incidence was 89.6%. Most of the cancers were well-differentiated squamous cell carcinomas

Key words : BNUR, Bleomycin, Cu-bleomycin, Esophageal cancer of the rat, Polarizing microscopy,

Present address : The Send Surgical Division, Yamaguchi University School of Medicine, Ube, Yamaguchi, 755, Japan.

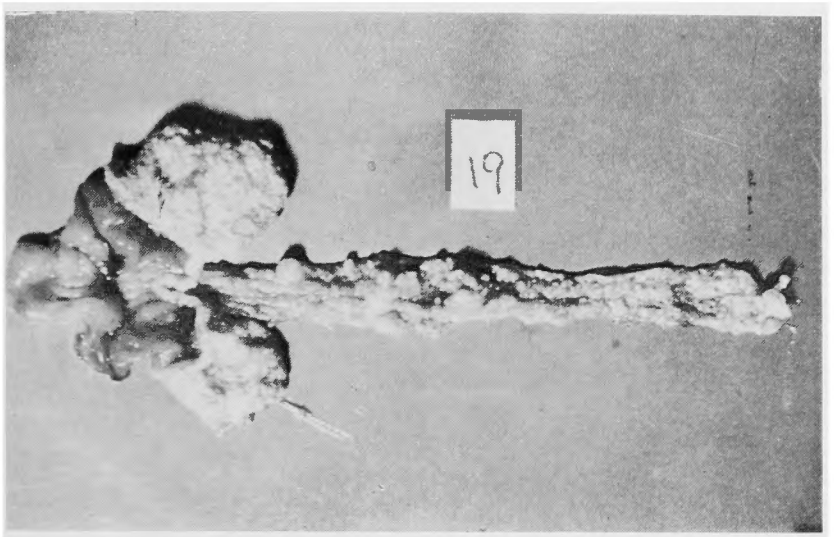

Photo. 1. BNUR-induced neoplasmas of the esophagus and the forestomach of the rats. Twenty-five weeks after administration of BNUR.

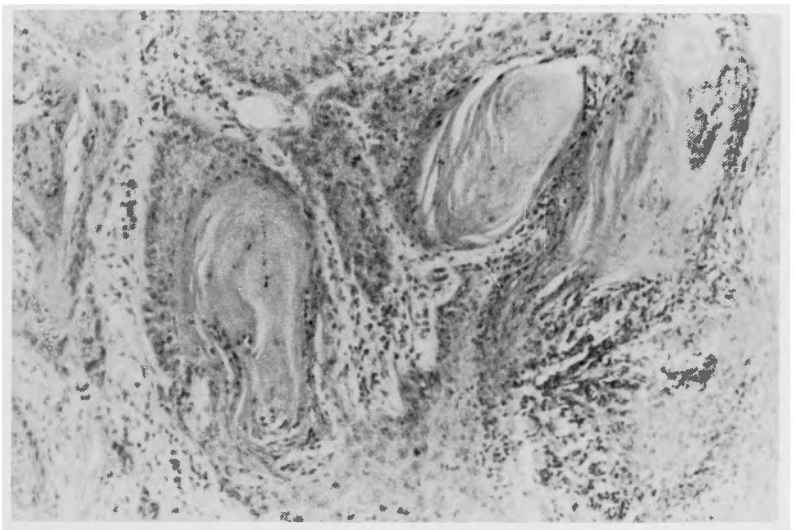

Photo. 2. The histology of the BNUR-induced keratinizing squamous cell carcinoma of the esophagus. Twenty-five weeks after administration of BNUR. Hematoxylin-eosin. (×100)

and showed keratinizing types (Photo. 2). Two rats had poorly-differentiated squamous cell carcinomas. None of the cancers showed a large tumor and infiltration into the mediastinum. Only one rat had the cancer which infiltrated to the adventitia of the esophagus. Because the cancer of the rat disturbs the swallowing at an early stage, the cancer cannot enlarge enough to infiltrate into the mediastinum. But in the forestomach, 3 rats had large tumors which were accompanied with a carcinomatous ulcer (Photo. 3), and the histology

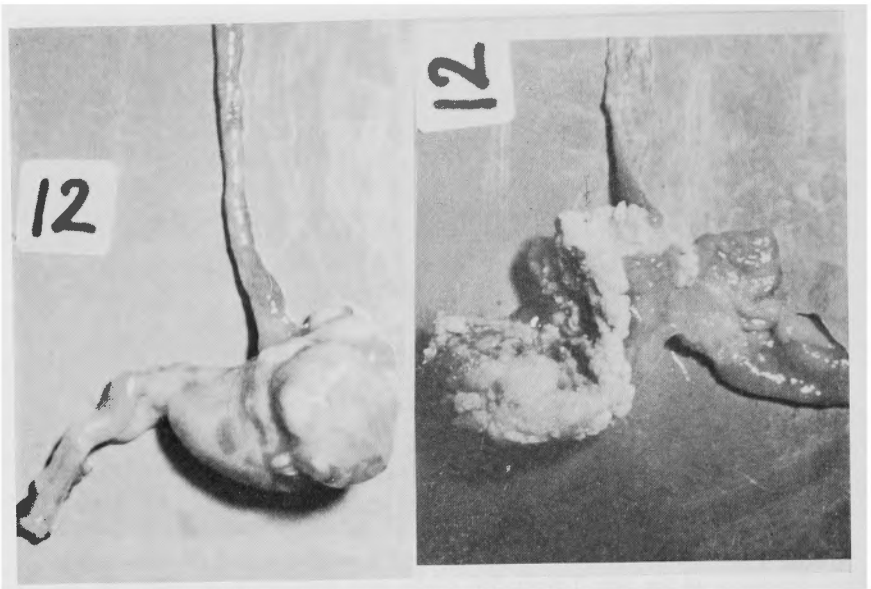

Photo. 3. Large tumor of the forestomach induced by BNUR. The tumor is accompanied with a carcinomatous ulcer. Twenty-eight weeks after administration of BNUR.

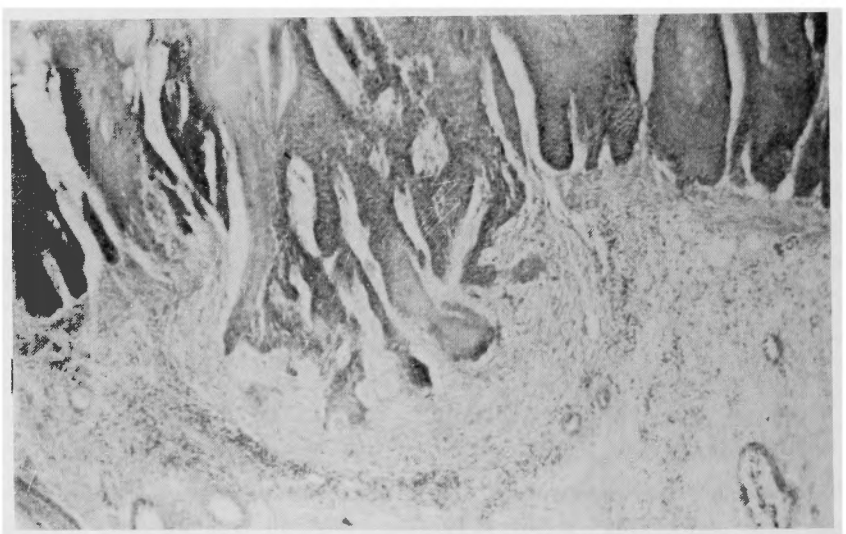

Photo. 4. The histology showed the squamous cell carcinoma infiltrating into the submucosal layer, that is, "down growth." Hematoxylin-eosin. (×40)

of these tumors were well-differentiated squamous cell carcinoma. The cancers in the forestomach showed a tendency to infiltrate toward muscularis mucosae, that is, "down growth" (Photo. 4). None of the BNUR-induced cancers showed metastasis toward local and distant lymph nodes and the other organs. One rat had a cancer infiltrating into the abdominal wall directly. One of the most prominent features of BNUR-induced cancer of

rats was multiple occurrence of squamous cell carcinoma extending from the esophagus to the forestomach.

(2) *The distribution of copper-containing BLM in BNUR-induced neoplasmas of rats.*

This experiment was performed with the histochemical method. Materials and methods: Twenty rats that received BNUR administration were sacrificed by exsanguination 60 minutes after intramuscular injection of copper-containing BLM (copper content : 3.42%) at a dose of 250 mg/kg. The esophagus and the stomach were removed as soon as possible after death. These tissues were stained with the modified silver sulfide method described by TIMM in 1958 (Table 1)[24].

Table 1. Histochemical demonstration of copper-containing BLM (Silver sulfide method by Timm.)

1) Fresh resected tissue is fixed by dehydrated alcohol saturated with sulfurated hydrogen sulfide.
2) Fixed tissue is washed dowh excess hydrogen sulfide by dehydrated alcohol.
3) Paraffine section is prepared.
4) After removing the paraffine, the preparation is washed by distilled water.
5) The preparation is stained with silvering by the following fluids between 30 and 60 min.
   A) fluid { 15~20% Gum arabic water solution : 10ml
   10% Silver nitrate water solution : 0.2ml
   B) fluid { Hydroquinone 2g, Citric acid 3g
   Distilled water 100ml
   All of A) fluid added to 2ml of B) fluid is used for staining.
6) Washing in distilled water.
7) Staining with hematoxylin.
8) Dehydration, transparency and enclosed by barsum.
   Results : Copper is stained and becomes brownish colored granules.

Results : Black brownish granules of copper were scattered in the tunica propria and submucosal layer of the esophageal wall (Photo. 5). In papillomas, copper-containing BLM distributed in the dendritic interstitial connective tissue (Photo. 6). In squamous cell carcinomas, these dark brownish granules were abundant in the connective tissue which surrounded the cancer cells (Photo. 7). Undoubtedly, copper-containing BLM distributed in greater amounts to the cancer lesions than to the normal esophagus and the papillomas. But the distribution in the well-differentiated squamous cell carcinoma did not differ from that in the poorly-differentiated type of carcinoma. Because the copper-containing BLM is decomposed into Cu and BLM at the time of intake into the cell, the distribution of copper-containing BLM in the cell cannot be observed by this histochemical method.

(3) *Observation of the neoplasms using the polarizing microscope.*

Materials and methods : The above-mentioned preparations were stained with hematoxylin-eosin and were observed under the polarizing microscope.

Results : Under the polarizing microscope, the squamous epithelium, such as the skin and the esophagus of the rat, showed the gleam in the cell and the keratosic layer, because the tonofibrils in the cytoplasm showed positive intrinsic birefringence and the keratosic

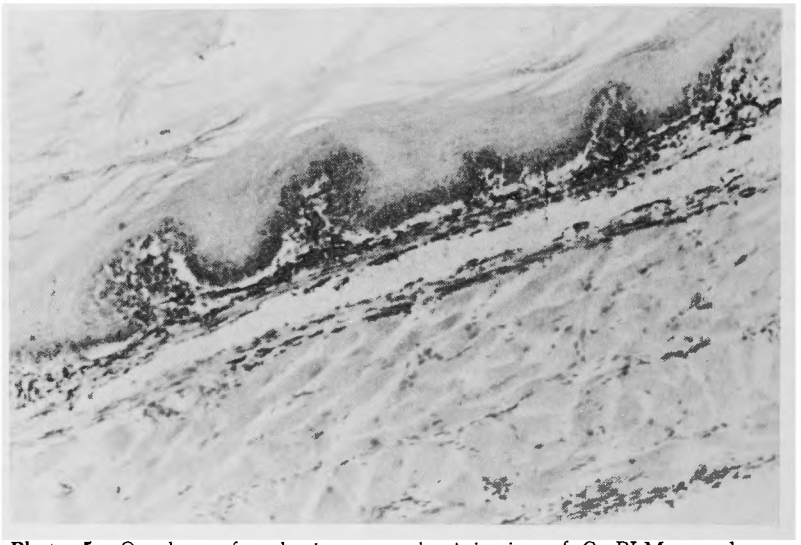

Photo. 5. One hour after the intramuscular injection of Cu-BLM at a dose of 250 mg/kg. The histology showed hyperplastic mucosa scattered with dark brownish granules of Cu-BLM in the tunica propria and submucosal layer. The stain was performed by the silver sulfide method by Timm. (×40)

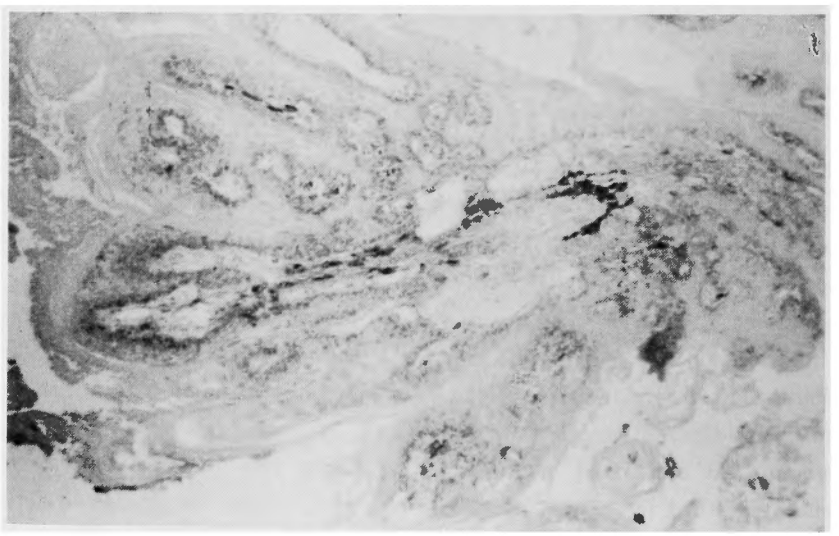

Photo. 6. One hour after the intramuscular injection of Cu-BLM at a dose of 250 mg/kg. The dark brownish granules of Cu-BLM were observed in the interstitial connective tissue of papilloma. The stain was performed by the silver sulfide method by Timm. (×40)

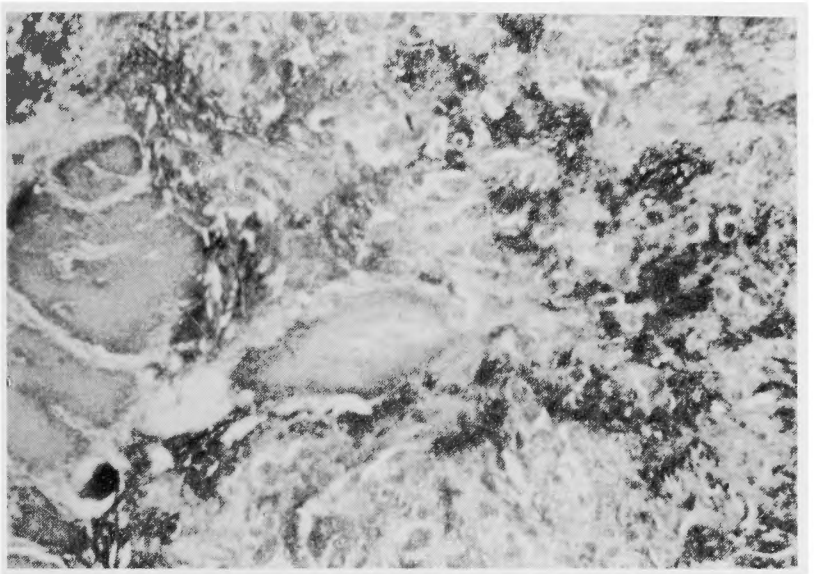

Photo. 7. One hour after the intramuscular injection of Cu-BLM at a dose of 250 mg kg. The dark brownish granules were observed abundantly in the interstitial connective tissue surrounding well-differentiated squamous cell carcinoma. The stain was performed by the silver sulfide method by Timm. (×40)

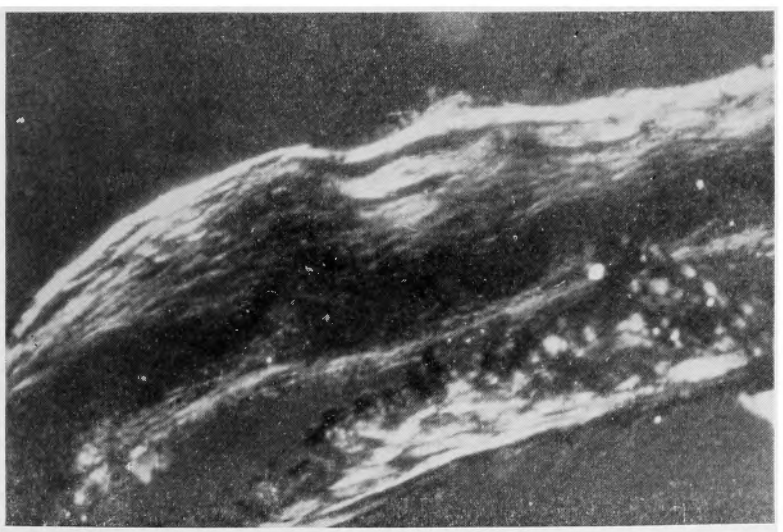

Photo. 8. The esophagus of the rat that received BNUR administration was observed under the polarizing microscope. The keratosic layer and the tonofibrils in the epithelium gleamed. Hematoxylin-eosin. (×40)

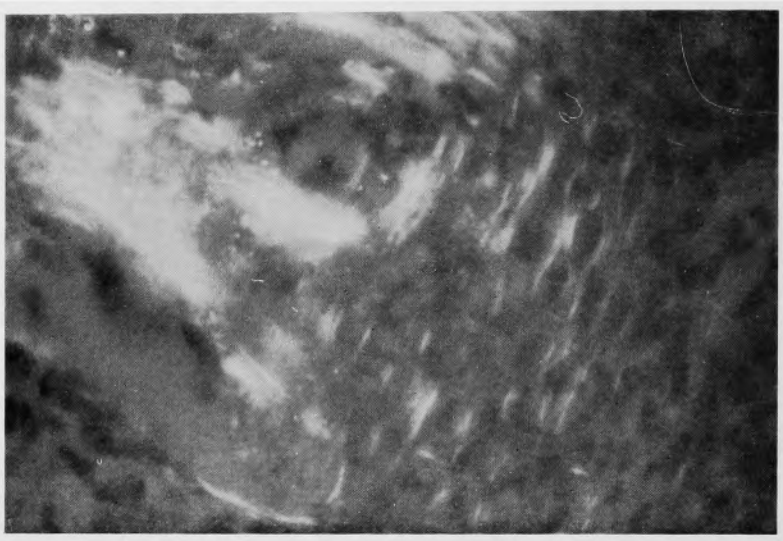

Photo. 9. The well-differentiated squamous cell carcinoma was observed under the polarizing microscope. The gleam of the tonofibrils in cancer cells kept the appearance of the squamous epithelium. The stain was hematoxylin-eosin. (×400)

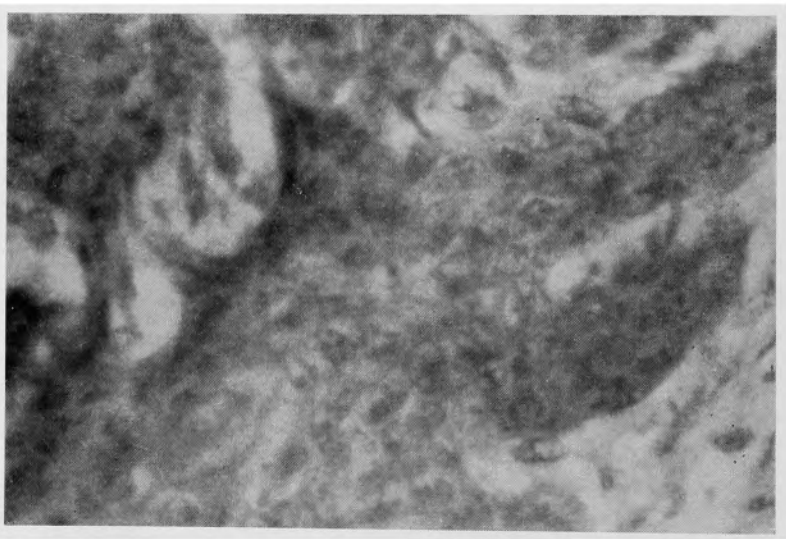

Photo. 10. The poorly-differentiated squamous cell carcinoma was observed under the polarizing microscope. The tonofibrils in cancer cells were observed in a small amount. Hematoxylin-eosin. (×400)

layer showed negative intrinsic birefringence[22]. In the esophagus of the rat, the gleam of the tonofibrils in the squamous epithelium increased gradually as the cells drew near the keratosic layer (Photo. 8). In the cases of BNUR-induced well-differentiated squamous cell carcinoma, the tonofibrils in the cancer cells kept the appearance of normal squamous epithelium, that is, it was increased in amount as the cells drew near the cancer pearl

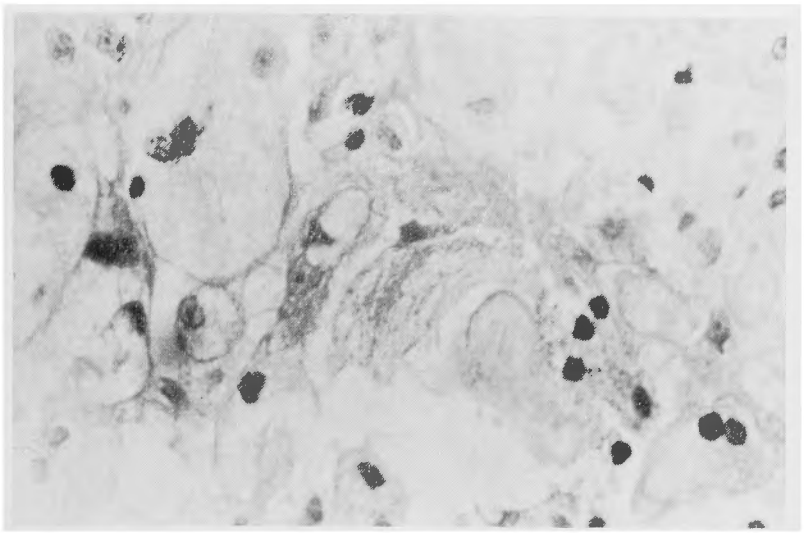

Photo. 11. The BNUR-induced squamous cell carcinoma treated with BLM for 8 weeks. Slight degenerative changes of the cancer were associated with the vacuolation and pyknosis of the nucleus and eosinophilic changes of the cancer cells. Hematoxylin-eosin. (×400)

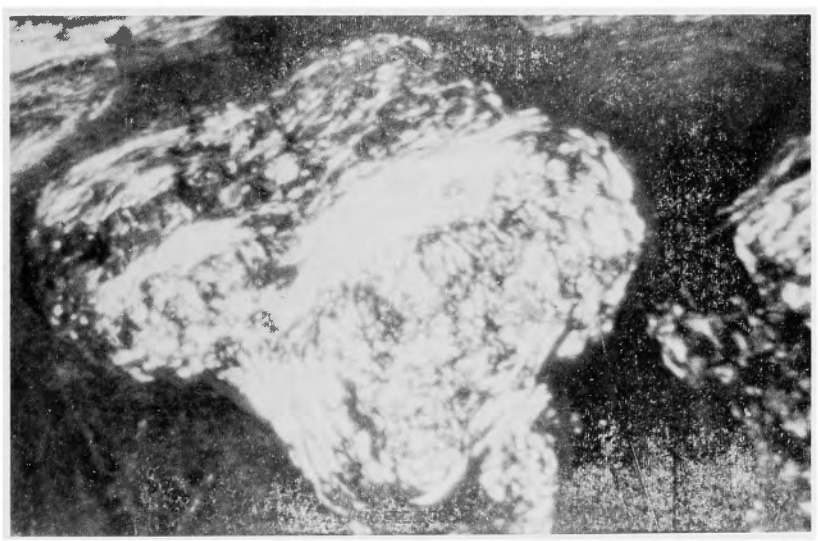

Photo. 12. The BNUR-induced squamous cell carcinoma treated with BLM for 8 weeks. The histology was observed under the polarizing microscope. The cancer pearls glittered more brightly. Hematoxylin-eosin. (×100)

(Photo. 9). On the other hand, poorly differentiated squamous cell carcinoma did not reveal such appearance as seen in normal squamous epithelium in the tonofibrils and contained only small amount of tonofibrils (Photo. 10).

(4) *Histological changes of the BNUR-induced cancer treated with BLM.*

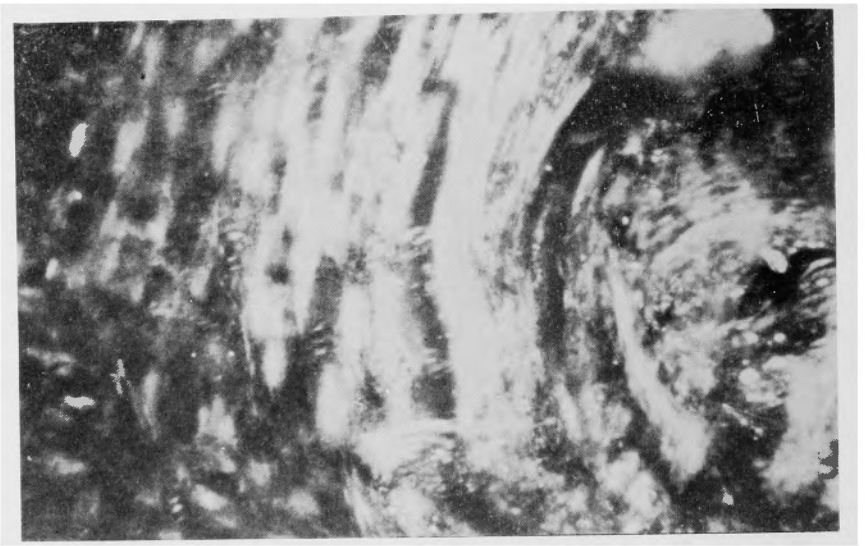

Photo. 13. The BNUR-induced squamous cell carcinoma treated with BLM for 8 weeks. The observation was performed by means of the polarizing microscope. The cells which were stained homogenously with eosin glittered brightly near the cancer pearls. Hematoxylin-eosin. (×400)

Materials and methods : Twenty weeks after administration of BNUR, nine rats continued to receive intramuscular injections of BLM at a dose of 2 mg/kg twice a week for 8 weeks. Thereafter, these rats were sacrificed and the esophagi and the forestomachs were stained with hematoxylin-eosin. The observation was performed by means of the light and the polarizing microscopes.

Results : The light microscopic observation showed that BLM was fairly effective on the BNUR-induced squamous cell carcinoma, although the vacuolation and pyknosis of the nucleus and eosinophilic changes of the cancer cells were observed in several places (Photo. 11). Under the polarizing microscope, the cancer pearls treated with BLM glittered more brightly than nontreated cancer pearls (Photo. 12). The cells which were stained homogenously with eosin glittered brightly under the polarizing microscope (Photo. 13).

Discussion

Recently, many carcinogens of the esophageal cancer of the rats have been reported by many authors[2)8)9)17)19)21)27)]. Most of these carcinogens are classified into nitrosocompounds, such as N-nitrosomethylvinylamine, N-nitrosoethylamylamine, N-nitrosoethylbutylamine, N-nitrosopiperidine and N-butyl-N-nitrosourethan (BNUR). TAKEUCHI reported that BNUR induced neoplasmas in the upper alimentary tract, showing squamous epithelium of the rats in high incidence[21)]. TAJIMA reported that the incidence of the esophageal cancer by BNUR in Donryu rats was 91.7% and these cancers were mostly of squamous cell type with variable differentiation from non-keratinizing to keratinizing variety[19)]. In our experiments, the

incidence of cancer in the esophagus and the forestomach was 89.6% and these cancers were mostly of well-differentiated squamous cell carcinomas and produced in many places of the esophagus and the forestomach. From the standpoint of multiple occurrence, these cancers in rats are different from cancers of the esophagus in man. Many authors have reported that BLM is more effective to well-differentiated squamous carcinoma[3)10)14)18)]. So, the author supposed that the distribution of BLM was more abundant in well-differentiated squamous cell carcinoma than the poorly-differentiated type. In the histochemical observation using copper-containing BLM, the drug distributed richly to cancer lesion than to the normal esophagus, but the difference of the distribution between both types of carcinoma was not observed. KANAO[12)], who studied the distribution of BLM in 20-methylcholanthrene-induced uterine cancer of the rats, mentioned that BLM distributed to the connective tissue surrounding the cancer cells remained unchanged for 24 hours. It is certain that BLM has an affinity for squamous cell carcinoma and remains in this tissue for a long time. There have been a few detailed descriptions about the histological alterations of the cancer lesion treated with BLM[1)16)18)19)20)23)]. According to AMANO, histological changes in the esophageal cancer induced by BLM therapy were swelling, pyknosis, vacuolation, dissolution and disappearance in the nucleus of cancer cell and vacuolation, swelling, eosinophilic changes and abnormal keratosis in the cytoplasm[1)]. Furthermore, the cells which were stained homogenously with eosin were scattered in the cancer lesion[20)]. These cells were the products of abnormal keratosis, because these keratosic cells were not stained in an ordinary color by the AYOUB-SKLAR method which stained keratosic substance. The cell was called "keratosic necrosis" by IKEDA who mentioned that the cells were the products of keratogenic action of BLM[10)]. The author observed many similar cells in BNUR-induced squamous cell carcinoma treated with BLM. These cells glittered brightly when observed with the polarizing microscope. These cells were observed near the cancer pearls of the well-differentiated squamous cell carcinoma treated with BLM. Moreover under the polarizing microscope, the cancer pearls treated with BLM glittered brighter than those in untreated case. These findings may provide some clues to the judgement of the results of BLM treatment. Recently, BLM effects on the esophageal cancer were divided into three grades[11)]. These gradings of effects are decided by subjective observation. The observation with the polarizing microscope may provide an objective conclusive factor of the effect of BLM treatment.

Conclusion

Twenty-nine Donryu rats were administered with N-butyl-N-nitrosourethan (BNUR) in drinking water at the concentration of 0.04% for 20 to 40 weeks. These rats which had various neoplasmas were investigated from various points of view. The results were as follows : 1) Neoplasmas induced by BNUR were papillomas and squamous cell carcinomas in the esophagus and the forestomach. The incidence of the papilloma was 100% and that of the cancer was 89.6%. Most of the cancers were well-differentiated squamous cell

carcinomas and only two rats had poorly-differentiated squamous cell carcinomas. These cancers showed no metastasis toward lymph nodes and the other organs. One of the most prominent features of these cancers was multiple occurrence of squamous cell carcinoma extending from the esophagus to the forestomach. 2) The distribution of copper- containing BLM in the cancer was richer than in the papillomas and the normal esophageal wall. The difference of the distribution between the well-differentiated squamous cell carcinoma and poorly-differentiated type could not be observed. 3) Under the polarizing microscope, the tonofibrils in the cell glittered. The tonofibrils in the squamous epithelium increased gradually as the cells drew near the keratosic layer. In well-differentiated squamous cell carcinomas, the tonofibrils in the cancer cells kept the appearance of the normal squamous epithelium. But in poorly-differentiated squamous cell carcinoma, the appearance of the tonofibrils did not keep that of the normal epithelium. 4) Bleomycine (BLM) was fairly effective on the BNUR-induced squamous cell carcinoma. After BLM treatment, various histological changes were observed, such as vacuolation and pyknosis of the nucleus of the cancer and eosinophilic changes of cancer cells. The latter glittered brightly under the polarizing microscope. The cancer pearls treated with BLM glittered more brightly than those in nontreated cases.

The author expresses gratitude to Prof. Dr. Koichi Ishigami for his kind guidance and to the staffs of our department for their cooperation throughout this study. Thanks are also due to Masataka Fujii, the director of Mitô Hospital, and Nihon Kayaku K. K. for their generous supply of Cu-BLM

The abstract of this paper was presented at the 77th Annual Meeting of the Japan Surgical Society, Tokyo, March, 1977.

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare.

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和文抄録

N-butyl-N-nitrosourethan (BNUR) によるラット実験食道癌および前胃部癌に関する研究

山口大学医学部外科学教室第2講座 (指導：石上浩一教授)

安 本 忠 道

BNUR によるラット食道および前胃部における腫瘍について種々の面より検討を加え，次のような成績を得た．

1) Donryu rat 29匹に BNUR を飲料水に混じて，20週～40週間投与した．全例に食道から前胃部にわたって，ほぼ均等に多発性の乳頭腫の発生を認めた．食道では所々に上皮の過形成や基底部の異型的増殖を認めた．癌は89.6％に発生し，ほとんどが多発性であった．組織学的には大多数が高分化型扁平上皮癌であったが，2例に低分化型扁平上皮癌の発生をみた．リンパ節転移や臓器転移を認めたものはなかったが，1例において前胃部腫瘤が直接腹壁に浸潤していた．食道では大きな腫瘤形成や縦隔に浸潤したものは認めなかったが，前胃部では大きな腫瘤を形成したものが3例に認められた．

2) 含銅ブレオの分布状態をみると，正常食道上皮では粘膜固有層から粘膜下層に多く分布し，乳頭腫では樹枝状の間質結合織内に多く認められた．癌病巣部では癌細胞巣をとり囲む間質結合織内に豊富に分布していた．明らかに正常組織や乳頭腫より的癌病巣部に多く分布していたが，高分化型と低分化型における分布の差は特に認められなかった．

3) 偏光顕微鏡的観察では上皮や癌病巣内の張原線維は長軸に positive な固有複屈折を示し，角化層は上皮表面に垂直な軸に対して強い negative の複屈折を示し，よく光った．この光は正常上皮では角化層に近づくに従って強くなり，張原線維が増量していることを示した．高分化型扁平上皮癌では正常上皮の形態を保ち，癌真珠に近づくに従って強い光を発したが，低分化型扁平上皮癌では張原線維は少く，このような規則性を示さなかった．

4) 9匹の Donryu rat に BNUR 投与20週目より BLM の 2mg/kg を週2回，8週間筋注し，その組織学的変化を観察した．癌細胞の空胞変性，核濃縮，細胞質の空胞変性，エオジンで均一に染る細胞の出現などの変化は認めたが，癌細胞全体では活動性が十分残っており，BNUR による Rat 扁平上皮癌に対して BLM は奏効しなかった．偏光顕微鏡による観察では BLM 投与例の角化層および癌真珠の複屈折は非投与例より強く，特に癌真珠周辺のエオジンで均一に染る細胞は強い複屈折を示した．この細胞は池田らのいわゆる“角化壊死”に相当し，BLM の角化促進作用の結果と考えられる．これらの偏光顕微鏡による観察は BLM 効果の観察の一助となり得ると考えられる．