Group	Treatment	N° of animals	Tba*	Histologically verified Tumors	Papillomas	Squamous cell carcinomas	Regressing tumors
1	DMBA only	30	8	12	6	-	6
2	Poly AU before DMBA	30	18	21	11	-	10
3	Poly AU after DMBA	30	12	16	5	2	9

*Tba = tumor bearing animals.

statistically significant ($p > 0.05$), group 3 vs. group 1) using the χ^2 test. Histological analysis (table) of the tumors showed most tumors to be papillomas with some of them regressing prior to termination of the studies. The only malignant tumors were seen in animals receiving Poly AU after DMBA (group 3).

Previous studies have shown that poly AU enhances both intracellular levels of cyclic AMP[13] and immune responses[9-13]. Cyclic AMP, like Poly AU, stimulates DMBA carcinogenesis when administered with DMBA or preceding carcinogen treatment[14]. Recently, another polycyclic aromatic hydrocarbon carcinogen, benzo(a)pyrene, was shown to induce an antibody in mice. The presence of antibody stimulated tumor development[8]. The enhancement of tumor formation seen when the immune response is stimulated before or at the time of carcinogen administration may be due to heightened carcinogen antibody formation.

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An uptake of fluorescein isothiocyanate labeled neocarzinostatin into the cancer and normal cells[1]

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Summary. An uptake of fluorescein isothiocyanate labeled neocarzinostatin into normal and cancerous epithelial cells from bladder was investigated. Results showed that neocarzinostatin traversed the cell membrane into cytosol and nuclei, and it appeared to have a preferential cytotoxicity for the cancer cell.

Neocarzinostatin (NCS) is an unique proteinacious antibiotic of known amino acid sequence[2] and is used for cancer treatment in man[3]. It inhibits DNA synthesis in bacteria and mammalian cells and arrests mitosis[4,5]. Although most of the molecular mode of action of NCS have been clarified as DNA strand scission at primarily thymidinyl residue followed by phosphorylation at both 3′ and 5′ sites which result in the inhibition of DNA synthesis[6-10], it is still unclear at the cellular level whether NCS actually enters into the cell to exert its effects. We have investigated this point by means of fluorescence microscopy using fluorescein isothiocyanate labeled NCS (F-NCS) and tumor cells obtained from human bladder cancer (transitional cell carcinoma histologically) as well as normal cells.

Materials and methods. F-NCS was prepared as described previously and retained original biological activity[11]. Bladder tumor and normal bladder epithelium were taken from transurethral biopsy and/or surgery. The tissues were minced and treated with 0.25% trypsin for 30 min at 37 °C. After filtration through a platinum mesh (No. 80), freed cells were washed twice with 0.01 M phosphate buffered 0.15 M saline (PBS, pH 7.0) and subcultured in RPMI 1640 supplemented with 10% foetal calf serum for 4 h to allow repair of the damaged membrane. Then, cells were washed twice with glucose Hanks' balanced salt solution (pH 7.2) and suspended in it to give a concentration of 10^6/ml. Aliquots of 0.5 ml of cell suspension were mixed with 0.2 ml of F-NCS (OD490 nm = 0.165) and incubated at 37 °C. After varied intervals, a small drop of cell suspension was placed on a slide glass and examined by a fluorescence microscope directly. In order to verify the incorporation by cancer cells, cells incubated with F-NCS for 1.5 h were washed 3 times with PBS, fixed with ethanol and then xylene, and embedded in solid paraffin followed by slicing with microtome into thin section (5 μm). The sliced thin section was placed on a slide glass and washed with xylene to remove paraffin before fluorescence microscopy.

Results and discussion. During initial 30 min, an uptake of F-NCS was slow and not distinct. Fluorescence was observed as sparse granules in cytoplasm as well as in nuclei. An enhanced incorporation of F-NCS by cancer cells was observed after 1 h. After 1.5–2 h of incubation with F-NCS, the uptake reached a maximum. As shown in figure 1, apparent fluorescence was observed in cytoplasm as well as in nuclei. Furthermore, in the cytoplasm, F-NCS was distributed homogenously and its uptake was thus definite. Furthermore, by examining the sliced sections, the uptake of F-NCS was also confirmed both in cytoplasm and nuclei, although it was less distinct than that of unsliced materials (figure 2). When uptake was compared with that of normal cells, the incorporation by the tumor cells was higher and faster than that by the normal blood

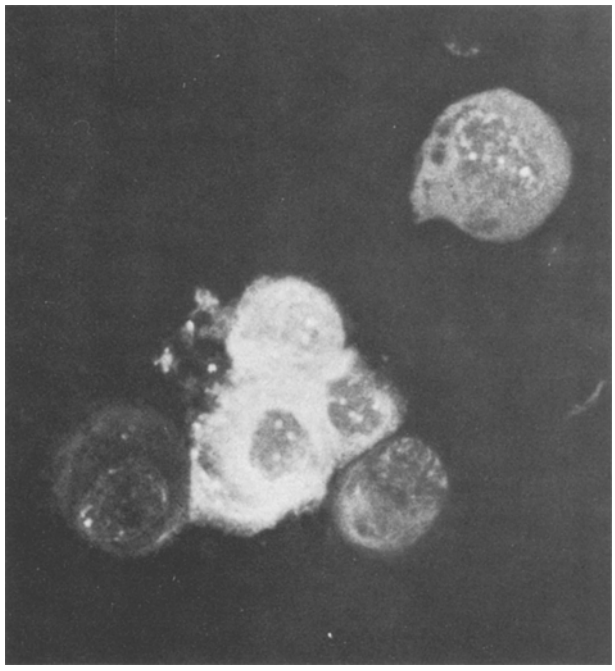

Fig. 1. Fluorescence micrograph of bladder cancer cells after 1.5 h incubation with F-NCS. Note clear fluorescence due to F-NCS in cytoplasm as well as nuclei. Original magnification × 400.

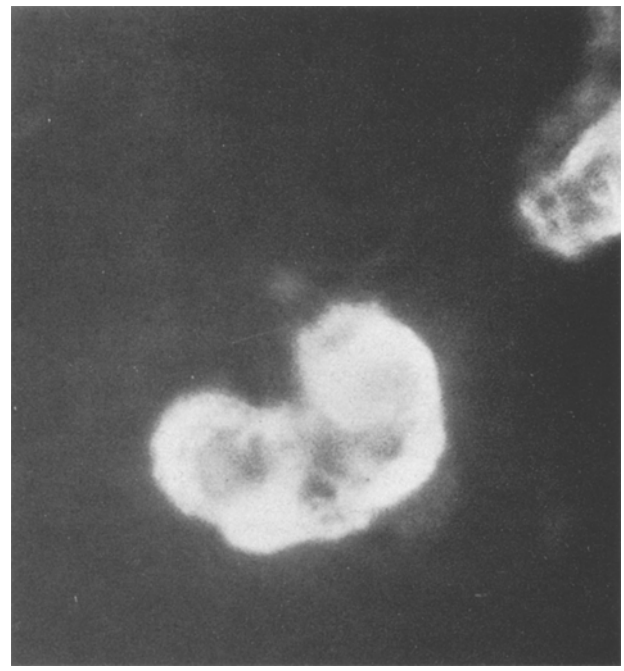

Fig. 2. Fluorescence micrograph of sliced section of bladder cancer cells. Fluorescence due to F-NCS distributed homogenously in the cytoplasm. Original magnification × 1000.

cells (lymphocyte or erythrocyte) or by the normal epithelial cells. These normal cells did not incorporate the F-NCS, or incorporated very little and that slowly. Moreover, non-viable cancer cells, as judged by trypan blue dye exclusion method, did not incorporate F-NCS. Degeneration of cancer cells such as swelling, vacuolation and bleb formation were observed after 4 h of treatment under fluorescence or light microscopy (figure 3). In the normal cells, however, the degenerative changes could not be recognized in 5 h.

The known molecular mechanism of NCS favors its direct action on DNA rather than indirect action. However, Ebina et al.[12] described that NCS affected microtubular proteins on cell membrane and thus regulated DNA synthesis. Lazarus et al.[13] showed that NCS is able to exert toxic effect by interaction with cell surface receptor. On the contrary, Kappen and Goldberg[14] reported that cell membrane is not required for the action of NCS. Namely, isolated HeLa cell nuclei were more susceptible to NCS than whole intact cells. We have demonstrated previously that [^{14}C] labeled NCS was not only taken up by lymphblastoid cells but also [^{14}C] labeled NCS was located on or in the isolated nuclei; as judged by radioautography[15]. These and present results show NCS penetrates the cell membrane and acts directly in the cells.

Viability of tumor cells as judged 10 h after the treatment was found less than 10%, while that of normal cells were more than 60% (not shown). Little uptake of F-NCS or no morphological changes in the normal epithelial cells may indicate selective incorporation (selective toxicity) in cancer cells. Furthermore, the selective cytotoxicity may be explained by the facts that the normal epithelial cells used were at the resting stage (G_0), and they undergo neither DNA synthesis nor cell devision at this stage, thus they were unaffected since it is known that NCS inhibits cell growth at G_2 stage[5,16]. In our separate studies employing lymphoblastoid cells (Epstein-Barr virus transformed cells) and peripheral lymphocytes (nondividing), the cytotoxicity of NCS was found more prominent in the transformed lymphoblastoid cells than normal lymphocytes[17]. These results are concordant with the present results.

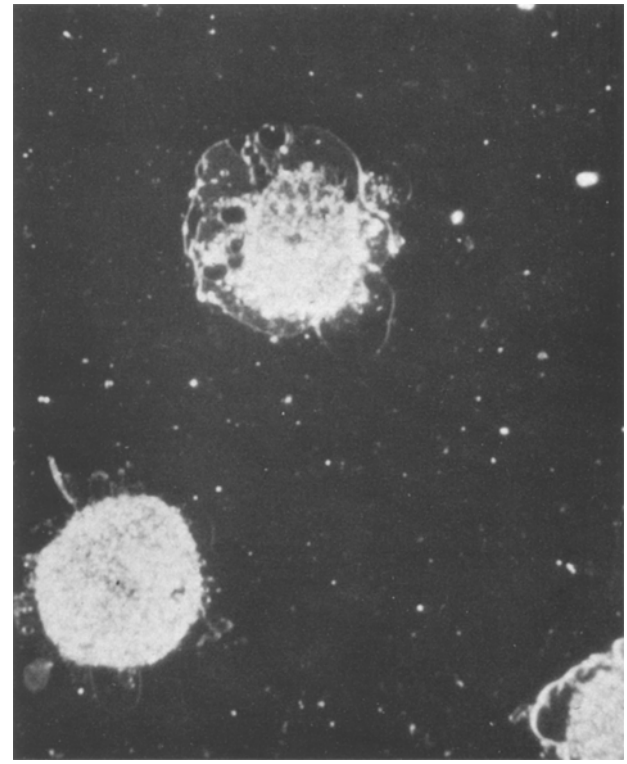

Fig. 3. Fluorescence micrograph after 4 h incubation with F-NCS. Degenerative changes such as vacuole, bleb and swelling were observed. Original magnification × 400.

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A histochemical study on the innervation of the cerebral blood vessels in the carp

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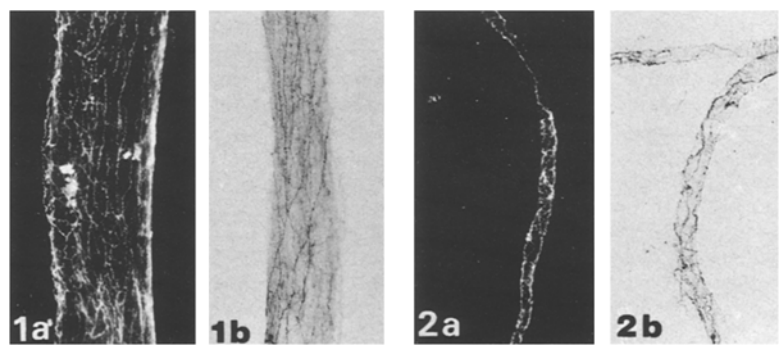
Summary. Adrenergic and cholinergic innervation of the cerebral blood vessels were studied in the carp using fluorescence and cholinesterase histochemistry. Most of the major cerebral and pial arteries were densely innervated with both nerves. Moreover, both nerves, being probably of central origin, were observed frequently on the walls of parenchymal arterioles and capillaries in the sections of the inferior lobe but were only rarely found in the other brain sections.

In the visceral and cardiovascular systems of vertebrates, the adrenergic fibres are a relatively late step in the vertebrate evolution[1]. However, recent comparative histochemical studies on innervation in the cerebral blood vessels support the assumption that the evolutionary trend for the autonomic nerves in the cerebral blood vessels is a unique one[2-7].

Material and methods. 35 carps, *Cyprinus carpio,* were used in this study. For whole mount preparation, the cerebral arteries were stretched on glass slides for fluorescence histochemistry, or were fixed with 4% formaldehyde buffered to pH 7.4 for 20 min for cholinesterase histochemistry. Small blocks of brain, frozen in dry-ice isopentane, were treated according to the freeze-drying or cryostat technique in order to demonstrate the innervation in the parenchymal vessels. For demonstration of adrenergic nerves, air-dried or freeze-dried materials were treated in formaldehyde vapor from paraformaldehyde for 1 h at 80 °C.[8] As for the cholinergic innervation, formaldehyde-fixed whole mount preparations or cryo-cut sections (20 µm) were maintained in substrate (acetylthiocholine-iodide)-free Karnovsky's medium[9] for 30 min at 4 °C, and then incubated in the complete medium, containing 2×10^{-4} M iso-OMPA as an inhibitor of nonspecific cholinesterases, for 1 h at 20 °C.

Results. Although the nerve fibres were rather fine as compared with those found in the other animals, most of major cerebral and pial arteries were innervated with dense adrenergic nerve plexuses (figure 1, a). Even the small pial arteries, especially those on the optic lobe, were also densely innervated with adrenergic fibres (figure 2, a). Although the AChE-activity of the nerve fibres was weak in comparison with that in mammals, the cholinergic nerve plexuses in those arteries showed almost the same density as those of adrenergic (figures 1, b and 2, b). The parenchymal arteries just penetrating into the brain parenchyma were accompanied by the adrenergic nerve plexuses (arrows in figure 3) continuing with those of the pial arteries. Moreover, the adrenergic nerve fibres and varicosities could be seen on the walls of arterioles and capillaries (arrows in figures 4, a–c), and a direct connection by the adrenergic nerves between the brain parenchyma and the walls of those small parenchymal vessels was sometimes observed (arrowhead in figure 4, a). This feature of the adrenergic innervation in the small parenchymal vessels was found frequently in the cross sections of the inferior lobe, but only rarely in the other brain sections. Some of the penetrating arteries within the brain parenchyma were also accompanied by the cholinergic nerves (arrow in figure 5). The cholinergic innervation in the parenchymal arteries, however, was much scarcer than that in the pial arteries of the same diameter and than the adrenergic innervation in the parenchymal arteries. The AChE-positive nerves contacting with the arterioles and the capillaires



Figs. 1 and 2. The adrenergic (a) and cholinergic (b) innervation in the posterior ramus of cerebral carotid artery (1) and the small pial artery on the optic lobe (2). a × 67; b × 64.