Preliminary Virological and Immunological Studies of the Salivary Gland Tumor of C3H/He Mouse

About 5 years ago we isolated a tumor histogenetically originating from the submaxillary gland in a C3H/He[1] mouse of our breeding.

It proved possible to transfer this tumor into isogeneic animals by means of cellular transplants[2], and this has now been done 112 times. During this time, the characteristics of progression of this tumor remained unchanged and, if examined comparatively to the submaxillary gland, it represents an excellent model for a study of the alterations occurring after the neoplastic transformation.

In previous works the morphology[3] and the metabolic properties[4] of the tumor have been reported. In this note we will describe the preliminary results obtained in a series of experiments carried out with the purpose of discovering whether a virus is the original cause of the neoplastic transformation.

The first point was to establish whether it was possible to isolate an agent identical or similar to the polyoma virus from tumoral cells after several transplantations in vivo. In this connection it is well known that most tumors of mouse parotid and other salivary glands are induced by this virus. For this purpose, mouse embryo cell cultures of C57BL/6 were used, being considered the most sensitive to the specific cytopathic effect of polyoma virus[5] and giving at the same time early evidence of a high hemagglutinating (HA) titre[6,7]. Experiments were carried out by adding the cellular suspension and the cell-free tumor extract to mouse embryo cell cultures. During a period of 35 days, the culture liquids, removed once a week, were tested for their HA titre in comparison with guinea-pig red blood cells at 4 °C for 60 min, and comparative examinations were carried out on the cytopathic effect (CPE) specifically induced by polyoma virus. Culture liquids were added to cultures of C57BL/6 mouse embryo cells or were inoculated into new-born mice of Swiss strain.

It is interesting to point out that we have been unable to observe any cytopathic effect after addition of cellular suspensions or cell-free tumor extracts. In addition, the HA titre of culture liquids never exceeded the value of 1:4 and no complement-fixing (CF) activity was found. As shown by the data reported in Table I, none of the newborn animals inoculated with culture liquids developed tumors in spite of continuous observations carried out for about a year. The cell-free extracts of tumor grown in vivo behaved similarly and a very low HA and a negative CF activity were observed.

On the basis of such results we can reject the hypothesis that the polyoma virus or a polyoma-like agent is to be considered responsible for the neoplastic transformation of submaxillary gland cells. A further confirmation has been obtained by evaluating the titre of hemagglutination-inhibition (HAI) antibodies in animals before, during and after the growth of the transplanted tumor. In 108 subjects which were examined, the antibodies titre remained at a constantly low level (below 1:40), though the observations were carried out from 20 days after the transplantation to a moment before the death.

In another series of experiments we studied whether the preventive inoculation of polyoma virus into animals, protected them from a successive challenge by whole tumoral cells. 0.2 ml polyoma virus suspension, corresponding to 160 HA units, were injected into 6 groups of C3H/He adult mice with a HAI titre below 1:40. After 24 days whole tumoral cells were inoculated. 30 days after the inoculation a 100% positivity was found and the characteristics of tumor development appeared exactly identical to those grown in the animals of the control group. In the tumor-bearing animals an average HAI titre of 1:640 was found.

The tumor growth in the animals inoculated with polyoma virus was maintained by means of a series of passages in vivo through successive subcutaneous transplants, and a new tumor cell line, further called C3H/Py, was derived. As shown by data of Table II, in the adult C3H/He mice injected with homogenized C3H/Py tumor cells the resulting HAI titre was 1:80 before challenging with whole cells derived from the 2 different tumor lines.

[1] This strain is derived from the original colony of Dr. STRONG, later sent to the Netherland Cancer Institute, to Gif at 22nd inbred generation in 1956, to ARSAL at 19th inbred generation in 1963, to Regina Elena in 1964. F: 22+19+3+1 at time of isolation of the tumor.

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[6] L. CASTELLI and A. M. JEMOLO, Oncologia *18*, 21 (1964).

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Table I. Attempts to recover polyoma virus in adenocarcinoma tumor cells by evaluation of CPE, HA and CF

Day of inoculation	C57BL/6 mouse embryo cell cultures, inoculated with: Homogenized tumor cells			Cell-free tumor extracts		
	CPE	HA titre	CF titre	CPE	HA titre	CF titre
7[a]	0	<1:4	<1:2	0	<1:4	<1:2
14[a]	0	<1:4	<1:2	0	<1:4	<1:2
21[a]	0	<1:4	<1:2	0	1:4[a]	<1:2
28[a]	0	<1:4[a]	<1:2	0	<1:4[a]	<1:2
35[a]	0	<1:4[a]	<1:2	0	<1:4[a]	<1:2

[a] 0.1 ml of the culture liquids were inoculated into new-born Swiss strain (total of 14 litters: 119 animals). After 285 days none of the surviving animals showed tumor growth (0/109).

Table II. Protective action of homogenized tumor cells after the addition of polyoma virus[a]

50 (3-month-old) C3H/He mice inoculated with homogenized cells of C3H/Py$_5$ tumor (0.5 ml 20% w/v)	
After 45 days (HAI titre for polyoma virus in inoculated mice serum 1:80)	
Challenge of 25 animals with salivary gland tumor cells (400,000 cells)[b]	Challenge of 25 animals with C3H/Py$_6$ tumor cells (400,000 cells)[b]
After 18 days	
Tumor takes 22/23 (95.6%)	Tumor takes 12/24 (50%)
After 30 days	
Tumor takes 23/23 (100%)	Tumor takes 20/24 (83.3%)

[a] Polyoma virus strain 210-877/4 from Dr. B. EDDY. [b] The dose of 4×10^5 cells was chosen assuming that the value at 4×10^4 gives 100% of tumor takes, evaluated at the 30th day after inoculation.

A temporary and mild protective action was only observed after challenging with whole tumor cells carrying polyoma virus. The slight protection decreased progressively as a function of the time, and after 45 days the percentage of tumor takes was essentially the same for both the animal groups.

In another series of investigations it was determined if the tumor was able to produce an appreciable immunological response in isogeneic hosts. 3 different types of experiments were carried out: 1) 4×10^5 trypsinized tumor cells were inoculated into adult C3H/He mice at the dorsal surface of the cartilage plate toward the distal edge of the ear or the extremity of the tail. As soon as the tumor reached an adequate development and before it could cause infiltration into neighbouring tissues, a radical excission was carried out and a challenge was made with isogeneic cells. 2) Adult C3H/He mice were inoculated with homogenized tumor cells and 30 days after a challenge with whole cells was carried out. 3) Adult C3HeB/FeJ mice, born from foster-nursing C3H/He suckled by C57BL/6 and therefore 'theoretically' free from Bittner virus, were incoulated with homogenized cells and 30 days after a challenge with whole tumor cells was carried out.

As shown by data of Table III, positive takes were obtained in all cases, thus indicating that it is impossible to induce any protection in the isogeneic host, at least with the methods employed.

In conclusion, from the data collected until now and briefly described here, 2 points must be emphasized. Firstly, the absence of virus particles and the specific cytopathic effect clearly indicates that mouse salivary gland tumor does not carry polyoma or polyoma-like viruses. In addition, when polyoma virus is artificially introduced into the system a slight decrease of tumor takes was obtained, further confirming that the etiology of this tumor cannot be restricted to polyoma virus. Secondly, it seems apparent that during its development the salivary gland tumor is unable to induce an appreciable immunological response.

Table III. Evaluation of immunological response in relation to the growth of the salivary gland adenocarcinoma in isogeneic hosts

No. of animals	Sites of inoculation	Growth	Days of challenge	Tumor takes 30 days after the challenge
23 C3H/He	Ear cartilage	100%	12[a]	100%
20 C3H/He	Tail	100%	12[a]	100%
30 C3H/He	Neck, s.c.	–	30[b]	100%
23 C3HeB/FeJ	Neck, s.c.	–	30[b]	100%

[a] From the removal of primary tumor. [b] From the inoculation of homogenized cells.

Riassunto. Dai dati riferiti si mettono in evidenza due punti: il primo dimostra nel tumore in esame l'assenza di particelle virali e di effetto citopatico specifico per il virus polioma. Inoltre non si mettono in evidenza anticorpi fissanti il complemento o emoagglutinoinbenti per il virus polioma negli animali portatori di tumori. Il secondo punto dimostra che durante il suo sviluppo il tumore della ghiandola salivare é incapace di indurre una risposta immunologica apprezzabile.

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Histone-Induced Macrophage Disappearance Reaction in Normal Guinea-Pigs

The complex state of delayed type hypersensitivity is represented by different types of specific reactions: The macrophage disappearance reaction (MDR)[1-6,13] and the release from cells of the lymph node permeability factor (LNPF)[7,8] should be cited as examples. A complete functional identity of histones from different sources and LNPF has been demonstrated previously[9,10]. It was of interest to examine whether or not a MDR could also be induced when inoculating normal guinea-pigs with calf thymus histone, instead of treating immunized animals (which exhibit delayed-type hypersensitivity) with the corresponding antigen.

Total histone from calf thymus nuclei was extracted with sulphuric acid (pH 0.7)[11,12]. The preparation was dissolved in saline, shifting the pH value to 7.0–7.2 and was injected s.c. or i.p. into either normal or BCG-sensitized guinea-pigs (Pirbright, 400–600 g). Histone solutions were sterilized by filtration prior to use. Peritoneal exudate was induced by glycogen (Schuchardt). 4 days later the peritoneal cells were harvested and the number of free floating macrophages in the peritoneal exudates was assayed by total and differential cell counts. All these methods, i.e. induction and harvesting of peritoneal exudates as well as differentiating macrophages, were accomplished in exactly the same manner as described by Nelson et al.[2,13].

Pilot experiments indicated that the average value of the total cell count showed much day-to-day variation in normal animals, as previously described by others[2,6]. However, a reliable evaluation of the disappearance of free floating macrophages was achieved when using the relative values of macrophages in differential cell counts only. Guinea-pigs were immunized s.c. by injecting 3 mg of BCG in 0.5 ml saline per animal. 3 or 4 weeks later, the animals were subjected to further experiments. A purified protein derivative of tubercle bacilli (GT, Hoechst) was used as tuberculin.

Intraperitoneal injection of tuberculin (GT) into BCG-sensitized guinea-pigs 2 h before cell harvesting resulted in quick disappearance of most of the free floating macrophages. With non-immunized animals this was not observed. These results are in agreement with the observations of Nelson et al.[1-3,13]. When histone was injected by this route, a marked disappearance of macrophages was observed, even with a dose of only 50 ng (see Table I).

Within 2 h after s.c. injection of a higher dose of 4 mg histone-N per animal, disappearance of macrophages in the peritoneal exudate was not observed, but a marked