Exencephaly in litters of foetuses from mice raised on tap or hard water

Maternal water supply	No. of mice	No. pregnant	Total implants	Total litter size	No. resorbtions	Normal foetuses	Exencephalic foetuses
Tap water (2.8 mg % Ca)	32	16 (50%)	98	6.1	32 (42%)	64	6 (13%)
Hard water (109 mg % Ca)	78	32 (40%)	240	7.9	94 (44%)	124	22 (18%)
Hard → soft at mating	48	10 (21%)	66	6.5	24 (36%)	26	16 (33%)

with lowered fertility (although litter size and number of resorptions were similar to those of controls) and more exencephalics.

In man, it has been suggested that neural tube defects such as anencephalus and spina bifida might be correlated with softness of drinking water[1-3], although Fielding and Smithells[4] were unable to demonstrate this correlation in South West Lancashire. In this short series in the mouse, a correlation does seem to be present, but in reverse, with more neural tube defects in the offspring of mice exposed to hard water. The effect of transfer from hard to soft water at mating, designed to test whether the effect was upon the foetus or the maternal physiology, seems merely to add an extra trauma.

Preparation of fixed antigenic, non-oncogenic and protective neoplastic cells

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Summary. Transplantable spontaneous mammary adenocarcinoma and sarcoma P 1798 cells were incubated with vibrio cholera neuraminidase (VCN), then fixed cells demonstrated ability to exclude trypan blue dye and to immunized animals and produce cytotoxic sera of high titers. The fixed neuramindase-treated tumor cells became non-oncogenic and protected the host against high doses of fresh untreated homologous tumors.

Experiments with in vitro assays have established[1] that most human malignant cells contain cell-surface antigens which are either absent or present at very low concentrations on the surface of normal cells of the corresponding adult tissue. Some of these antigens can evoke a specific cell-mediated immunity directed against malignant cells of corresponding tissue type[2]. Tumor cells treated with vibrio cholera neuraminidase (VCD) have been used to immunize mice[3,4] and to cause remission of small but progressively growing tumors[5-7]. Therefore, the present investigations were aimed at the preparation of fixed, antigenic, non-oncogenic cells that immunologically protect the host against spontaneous mammary adenocarcinoma and against sarcoma P 1798.

Materials and methods. Tumors Sarcoma P 1798 and spontaneous mammary adenocarcinoma were carried on in 2 strains, CFW_1 and BALB/c, 18–20 g inbred, brother-sister white mice. Doses of 10^3 viable sarcoma cells, or 10^4 viable adenocarcinoma cells administrered s.c., developed into solid tumors in all the inoculated mice and killed their hosts in 21–28 days. So far, no regression was observed in mice inoculated with each of these 2 strains.

Cell preparations. Transplantable mouse sarcoma P 1798 and spontaneous mammary adenocarcinoma cell suspensions in Eagle's minimum essential medium (MEM) were prepared from fragments of tumor tissue as described in an earlier communication[8]. For frozen cell preparation, cells were quickly frozen in liquid nitrogen. For vacuum dried cells, cells were quick-frozen in acetone-dry ice bath and dried under vacuum while frozen. Formalinized cell preparations were made by washing the cells with 0.85% NaCl solution and centrifuged at 1500 rqm for 10 min. 10 volumes of 2% buffered formalin were added to the packed cells which were suspended uniformly by gentle stirring at room temperature for 10 h. Cells were then washed twice with 0.85% NaCl solution and stored at 10°C until ready for use. The 2% buffered formalin contained per l 20 ml of 40% formaldehyde, 4g $NaH_2PO_4 \cdot H_2O$, 6.5 g Na_2HPO_4 and pH adjusted to 7.2. Glutaraldehyde cell preparations were made by washing the cells with 0.85% NaCl solution, then centrifuged at 1500 rpm for 10 min. 10 volumes of 0.2% buffered glutaraldehyde were added to the packed cells which were suspended uniformly by gentle stirring at room temperature for 1 h. Cells were then washed twice with 0.85% NaCl solution and stored at 10°C unitil ready for use. The 0.2% buffered glutaraldehyde was made in phosphate buffer and adjusted to pH 7.2.

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Table 1. Effect of various treatments on spontaneous mammary adenocarcinoma cells

Cell preparation	Intact cells (%) trypan blue	Cytotoxicity of immune serum***	Agglutinin required	Oncogenic capacity* at 10^4 cells	Protective capacity**
Fresh (control)	96 ± 5	1058 ± 98	185 ± 13	10/10	0/10
Frozen	23 ± 3	414 ± 27	115 ± 10	10/10	0/10
Freeze dried	22 ± 4	217 ± 17	296 ± 24	10/10	0/10
Neuraminidase (VCN) treated	98 ± 4	1976 ± 127	495 ± 38	2/10	8/10
VCN-treated-frozen	69 ± 5	1542 ± 98	146 ± 12	2/10	8/10
VCN-treated-freeze dried	61 ± 5	2276 ± 196	287 ± 25	0/10	10/10
Formalin (2%) treated	90 ± 6	988 ± 81	167 ± 13	2/10	5/10
VCN-treated-formalinized	98 ± 5	1773 ± 113	334 ± 28	0/10	10/10
Glutaraldehyde (0.2%) treated	99 ± 6	1887 ± 118	394 ± 31	1/10	6/10
VCN-treated glutaraldehyde	97 ± 7	3089 ± 135	387 ± 9	0/10	10/10

* Oncogenic capacity expressed as number of animals died from tumor over number of animals treated with 10^4 viable intact cells. ** Protective capacity is expressed as number of animals that survived a second challenge dose of 10^6 viable intact tumor cells over total number of animals treated. *** Cytotoxicity of serum is expressed as the reciprocal of that dilution of serum that kills 50% of the cells in the cytotoxicity assay[8].

Neuraminidase treatment. Vibrio cholera neuraminidase 500 units of enzyme/ml was used. 1 unit of enzyme activity is equivalent to the release of 1 μg of N-acetylneuraminic acid from a glycoprotein substrate at pH 5.5 at 37°C The enzyme itself is non-cytolytic[9] and can release sialic acid from both normal and malignant cell surfaces at physiological pH values[10,11]. The sarcoma or the mammary adenocarcinoma cells were incubated with neuraminidase (25 units/5 × 10 cells) at 37°C for 1 h. After incubation, the cells were washed 3 times in an excess of supplemented Eagle's medium and finally suspended in the same medium. In this communication the neuraminidase-treated cells are designated as VCN-sar or VCN-AdCa for the sarcoma or the mammary adenocarcinoma, respectively.

Immunization. Groups of 18–20 g of CFW_1 or BALB/c mice were immunized with 4 doses of 10^2 viable untreated sarcoma or adenocarcinoma cells, the neuraminidase-treated sarcoma or adenocarcinoma, or fixed cells given at weekly intervals for 4 weeks, and antisera were harvested by cardiac puncture 1 week after the last immunizing dose. After inactivation of complement by heating at 56°C for 45 min. Antisera were assayed for cytotoxicity against the sarcoma and adenocarcinoma viable cells. Protective capacity of the various cell preparations was assessed by injecting s.c. 10^3 cells, then 21 days later the animals received 10^6 viable adenocarcinoma or sarcoma cells. 30 days later, a second challenging dose of the viable fresh untreated tumor cells was given to the surviving animals.

Results and discussion. Various procedures were used to preserve the biological and immunological integrities of the surface of transplantable sarcoma P 1798 and spontaneous mammary adenocarcinoma cells. Fresh untreated and neuraminidase-treated tumor cells were frozen, dried and fixed with formalin and with glutaraldehyde. In earlier studies, treatment with neuraminidase was shown to increase the cell surface antigenic determinants[12]. Neuraminidase treated cells were less oncogenic, more protective against homologous tumors, and produced antisera with higher cytotoxic titers than the untreated cells. Both untreated or neuraminidase treated tumor cells, whether formalinized or fixed with glutaraldehyde demonstrated ability to exclude trypan blue dye. Cells subjected to freezing showed a markedly reduced capacity to withstand perfusion by the dye. Therefore, formalin- or glutaraldehyde-fixed cells maintain gross membrane structure to the extent that they are capable of excluding trypan blue.

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Table 2. Effect of various treatments on sarcoma P 1798 cells

Cell preparation	Intact cells (%) trypan blue	Cytotoxocity of immune serum***	Agglutinin required	Oncogenic capacity* at 10^4 cells	Protective capacity**
Fresh (control)	98 ± 5	1273 ± 112	172 ± 12	10/10	0/10
Frozen	28 ± 3	398 ± 25	98 ± 8	10/10	0/10
Freeze dried	27 ± 5	197 ± 3	218 ± 21	10/10	0/10
Neuraminidase (VCN)-treated	99 ± 4	2089 ± 132	478 ± 35	3/10	7/10
VCN-treated-frozen	76 ± 7	1876 ± 116	131 ± 10	4/10	7/10
VCN-treated-freeze dried	68 ± 5	2368 ± 147	327 ± 26	0/10	10/10
Formalin (2%) treated	88 ± 5	1087 ± 85	158 ± 15	4/10	6/10
VCN-treated-formalinized	95 ± 5	1687 ± 107	375 ± 32	1/10	9/10
Glutaraldehyde (0.2%) treated	98 ± 5	1768 ± 114	427 ± 36	3/10	7/10
VCN-treated-glytaraldehyde	99 ± 5	2203 ± 123	527 ± 48	1/10	10/10

* Oncogenic capacity is expressed as number of animals died from tumor over number of animals inoculated with 10^4 viable intact tumor cells. ** Protective capacity is expressed as number of animals survived a second challenge dose of 10^6 viable intact tumor cells over the total number of animals treated. *** Cytotoxicity of serum is expressed as the reciprocal of that dilution of serum that kills 50% of the cells in the cytotoxicity assay[8].

Data in table 1 for the spontaneous adenocarcinoma and table 2 for the sarcoma P 1798 indicate that, at least with respect to cytotoxicity the fixed cells provoke a similar immune response. High titers against the adenocarcinoma or the sarcoma cells were obtained from animals immunized with either formalin or glutaraldehyde fixed cells. In contrast immunization with frozen or freeze dried untreated cells led to much lower cytotoxic titers. The titers obtained after immunization with fixed cells suggest that their immunological potential is not compromised and hence that their immunologic determinants are largely intact. These fixed cells are clearly not viable cells, yet they maintain immunogenecity to a far greater extent than the cells rendered non-viable by freezing and subsequent thawing or by freeze drying. This suggestion is also supported by the fact that the fixed cells were non-oncogenic and protected against large doses of viable untreated homologous tumor cells.

The above evidence suggests that formalin or glutaraldehyde fixation of neuraminidase treated cells results in cell preservation without alteration of antigenecity. Although the use of liquid nitrogen freezers for slow cooling, with concurrent use of preservatives such as dimethyl sulfoxide[13] may result in immunizing cell preparations of equivalent efficacy to that of fresh cells, formalin or glutaraldehyde fixation of neuraminidase-treated malignant cells is a simple technique. In addition, with respect to animal or human tumors, a stock of cell can be harvested whenever the tumors are available, or at a specific point in time, treated immediately with neuraminidase, fixed with either formalin or glutaraldehyde, and used as a constant source of immunizing cells with avoidance of possible immunogenic alteration of cell lines maintained in culture. The use of neuraminidase-fixed tumor cells as a source of antigen in detection of autologous immunoglobulins or in the early detection of neoplastic proliferation is under investigation.

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Induction of malignant skin tumors in mice by topical application of ethylnitrosourea[1]

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Summary. A high percentage of squamous cell carcinomas were induced in the skin of Swiss-Webster mice by repeated topical application of ethylnitrosourea, indicating that this nitrosamide possesses a potent carcinogenic action when topically applied.

With the exception of methylnitrosourea (MNU)[4], most otherwise carcinogenic nitroso-compounds have not been proven to be effective skin carcinogens in laboratory animals[5–7], when topically applied, despite the fact that these substances do not require any metabolic activation to exert their activity[7]. We report the results of a sucessful attempt to induce malignant tumors in mouse skin by repeated application of 1-ethyl-1-nitrosourea (ENU) directly on skin.

52 randomly bred 8-week-old Swiss-Webster mice (27 males and 25 females) were housed by sex in groups of 10 in clear plastic cages on San-i-cell bedding. They were given Wayne pelleted diet (Allied mills, Chicago, Illinois) and tap water ad libitum and were maintained under standard hygienic conditions (constant temperature: 22 ± 2 °C and humidity; 12 h of artificial light per period of 24 h). The interscapular region was shaved (2.5 cm^2) and 33.4 µl of a 1% solution of ENU (0.334 µg) in pure distilled acetone (free of contamination by polycyclic aromatic hydrocarbons-PAH) was applied topically twice a week for 20 consecutive weeks with an automatic dispenser (Hamilton Co., Whittier, Calif.). The total dose of ENU received by each individual animal was 13.34 mg.

The solution of ENU in acetone was prepared freshly immediately prior to each treatment (pH 6.8–7.2 at room temperature). The animals were then observed daily for appearance and recording of tumors. They were allowed to die spontaneously or were killed when moribund. All were autopsied and all tissues and tumors were observed microscopically.

A group of 80 mice (40 males and 40 females of same age and origin) were used as untreated controls and maintained under the same conditions as the test animals. A total of 34 skin tumors (65.4% of all animals) were ob-

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Sex	Total No. of skin tumors	No. of squamous cell carcinomas	Δ % squamous cell carcinomas[a]	Survival time (weeks)[b] Treated	Controls	Δ % survival time[c]
Males	21 (77.8%)[d]	16 (59.2%)[e]	+ 23.4%	43.5 ± 12.7	81.0 ± 17.4	−46%
Females	13 (52.0%)[d]	12 (48.0%)[e]		53.7 ± 15.3	89.0 ± 21.8	−40%

[a] % increase of squamous cell carcinomas in males over females. [b] Mean ± SE. [c] Percent decrease in survival time of treated over controls. [d], [e] Percent of total number of animals in the group.