EFFECT OF LOW TEMPERATURES ON THE BITTNER VIRUS OF MOUSE CARCINOMA

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In the *British Medical Journal* of March 26 (p. 511) appeared an Imperial Cancer Research Fund lecture by Professor W. E. Gye dealing with the propagation of mouse sarcomas by means of frozen and dried tumour tissue. The experiments described make it clear that by refrigeration and subsequent drying at a low temperature the tumour cells can be killed and an active agent liberated which is capable of starting tumours afresh, and which, judging from those of its properties so far known, is viral in nature. This holds good for both chemically induced and spontaneous sarcoma in mice, so that we can regard the virus as the continuing cause of sarcoma (i.e., the agent which is responsible for the continuing cell division), while the chemical in those tumours apparently started by it acts as the inciting cause, allowing of, or producing, the change in the virus from the latent to the active form.

This sequence of events can be closely paralleled by what is already known to be true of mammary adenocarcinoma in mice. Bittner and his co-workers in the United States have shown that these tumours have for their continuing cause a virus (the Bittner milk factor), but that this virus requires a chemical inciter (oestrin) before it can change from the latent to the active form and produce a cancer. This virus, of which the active form can be seen in the tumours with the electron microscope (Porter and Thompson, 1948) is present in the milk of lactating female mice in the latent form and enters the bodies of the young mice as they suckle, especially if the milk is drawn into the respiratory passages (Begg, 1949). Here it circulates throughout the life of the mouse, reaching every organ which has a blood supply (Woolley, Law, and Little, 1941), but because of its high tissue specificity it is capable of producing tumours only in mammary cells and then only in the long-continued presence of oestrin.

In the male mouse, mammary tubules, imperfectly developed and without secreting acini, are present over large areas of the subcutaneous tissue, but even in high cancer strains male mice do not develop mammary cancer unless they are subjected to prolonged treatment with oestrin, when tumours occur as in the females—i.e., the virus changes from the latent to the active form. Hitherto no method of obtaining the active form of the virus outside the body or apart from living tumour cells has been discovered. That obtained free in milk is naturally in the latent form and is carcinogenic only after long-continued action of oestrin.

In the present experiments, however, it has been shown that by freezing mammary carcinoma tissue at -79°C . for long periods the tumour cells are killed and the Bittner virus liberated in its active form. It is then capable of initiating new tumours in male mice without oestrin as readily as in female mice, but only when injected into mammary tissue, thus demonstrating its selective infectivity. That these tumours produced with refrigerated tissue are not transplants but are true new infections is also demonstrated by the fact that there is an increase in the percentage of tumours obtained the longer (within limits) the

tissue is frozen. This suggests the slow liberation of the active virus and also perhaps the denaturing of an inhibitory substance by cold. Such inhibitory substances have already been demonstrated in the Shope papilloma and carcinoma of rabbits and in sarcomas of chickens. The present experiments were done with both sporadic mammary cancers of inbred mice and with the "63" carcinoma (Bashford's adenocarcinoma) of the hybrid M.R.C.-Hunt strain. Roughly equal numbers of males and females were used.

I. Experiments with Frozen Carcinoma Tissue Inoculated Subcutaneously in Male and Female Mice

The technique followed was that worked out in the Imperial Cancer Research Laboratory for the transmission of sarcomas. The tumour tissue was removed aseptically from the freshly killed mouse and minced finely in a Craigie mincer (Craigie, 1949). This alone should reduce its transmissibility, since mechanical disintegration of carcinomas was shown by Jensen in 1903 to reduce transplantability. It was then diluted with an equal volume of 5.3% dextrose solution in most cases and drawn up into a 5-ml. syringe, from which it was run into sterile ampoules (1 ml. in each). These were sealed, cooled rapidly to -79°C . by immersion in a solution of solid CO_2 in "cellosolve" (ethylene glycol monoethyl ether, B.D.H.), and stored in a "deep freeze" storage cabinet kept at -79°C . From this one ampoule at a time was removed as required. The contents were just thawed and, as soon as fluid, were drawn up in a small syringe and inoculated subcutaneously in the right mammary region of mice of the same strain as the tumour. The average dose was 0.05 to 0.1 ml. per mouse, which represents 0.025 to 0.05 ml. of tumour mince. It was noticed that the frozen tissue underwent a physical change, becoming more homogeneous, softer, and slightly sticky on prolonged freezing. This change also occurs in long-frozen sarcoma tissue.

The mice were then examined at intervals of three to four days and the time of definite appearance of tumours noted. Although the mice all came from high cancer lines they were used before they were old enough to develop sporadic tumours, and in all cases the tumours obtained were at the site of inoculation. It is to be remembered that when carcinomas are obtained in male mice known to carry the Bittner virus, by painting with oestrin, the tumours appear in a haphazard distribution and not at the site of painting.

Table I shows that 239 mice were inoculated and 154 tumours obtained. Tumours were obtained with equal ease in male and female mice, and the occasional omission of dextrose seemed to make no difference. The time of appearance of the tumours was related to their rate of growth; rapidly growing tumours appearing often within 10 days, and slowly growing ones being delayed from seven to 12 weeks. The stable "63" tumour appeared fairly constantly at four weeks, some mice in each batch showing it a little later or earlier than this.

If these figures are examined statistically it becomes apparent that there is a slight increase in virulence the longer the tissue is frozen. Thus, if the experiments are broken up into groups the percentages of "takes" shown in Table II are obtained. This is consistent with the gradual freeing of virus particles from an inhibitory substance, protein in nature, by continuing cold, and is not suggestive of survival of intact tumour cells. The arguments against cell survival on freezing to -79°C . in the case of sarcoma tissue have already been stated in other papers (Gye, Begg, Mann, and Craigie, 1949), but

TABLE I.—Frozen Carcinoma Inoculated Subcutaneously

No. of Expt.	Strain	Tumour	Diluent	Time Frozen	Inoculated	Tumours	Time of appearance
M77	MRC/H	63	Dextrose	15 min.	6	3	4 weeks
M78	R III	Sporadic	"	15 "	6	3	"
M70	MRC/H	63	None	15 "	6	3	"
M60A	"	"	Dextrose	1 hour	6	1	3 "
M65	"	"	"	1 "	6	4	4-5 weeks
M60A	"	"	"	3 hours	6	1	3-4 "
M65	"	"	"	3 "	6	1	5 "
M60A	"	"	"	6 "	6	4	4-6 "
M65	"	"	"	9 "	6	3	5 "
M60A	"	"	"	12 "	6	1	4 "
M65	"	"	"	12 "	6	2	5 "
M60A	"	"	"	24 "	6	0*	—
M65	"	"	"	24 "	6	3	4 weeks
G3A	R III	Sporadic	"	24 "	5	5	11 days
G3B	"	"	"	24 "	5	5	10 "
G3B	"	"	"	24 "	4	6	11 "
M53	S/A	"	None	24 "	6	4	4 weeks
M60A	MRC/H	63	Dextrose	2 days	6	5	4-6 "
M65	"	"	"	2 "	6	2	3-4 "
G3C	R III	Sporadic	Dext.-cysteine,	2 "	6	6	3-4 "
G3D	"	"	Dextrose	2 "	6	6	3 "
M65	MRC/H	63	"	4 "	6	2	3-6 "
M65	"	"	"	7 "	6	4	2-6 "
M53	S/A	Sporadic	None	7 "	8	7	4 "
G3E	R III	"	Dextrose	12 "	6	5	3 "
G3F	"	"	Dext.-cysteine	12 "	6	6	3 "
M53	S/A	"	None	14 "	8	7	5 "
M65	MRC/H	63	"	14 "	6	0†	—
M57	S/A	Sporadic	"	21 "	4	4	2 weeks
M72	MRC/H	63	Dext. added on thawing	26 "	3	0†	—
M57	S/A	Sporadic	None	28 "	4	4	3 weeks
M74	MRC/H	63	Dext. added on thawing	35 "	4	4	3 "
M57	S/A	Sporadic	None	37 "	3	1	4 "
M57	S/A	Sporadic	None	42 "	4	4	3 "
M59	MRC/H	63	None	55 "	6	3	2 "
M86	R III	Sporadic	Dextrose	82 "	3	3	1 week
G25	MRC/H	63	"	97 "	3	2	13 days
M90	R III	Sporadic	"	99 "	3	2	9 "
G21	C3H	"	"	113 "	3	2	2 weeks
M58	"	"	"	118 "	6	3	7 "
M63	"	"	"	119 "	3	3	7 "
M64	S/A	"	"	121 "	3	2	12 "
G8A	R III	"	"	138 "	6	6	2-3 "
G8B	"	"	"	138 "	4	5†	2-3 "
M90	S/A	"	None	182 "	3	3	9 days

* Technical error.

† † error.

‡ Two inoculations in one mouse.

statistical proof by numbers of successful inoculations is not so readily obtained in the case of the sarcomas, since the phenomenon of selective infectivity is not shown by them (see later), and tumours are obtained in practically 100% after freezing for any time after 15 minutes.

TABLE II

Time Frozen	No. Inoculated	No. of Tumours	Successes
15 minutes to 12 hours	72	30	41%
24 hours to 7 days ..	76	55	72%
12 to 182 days ..	91	69	75%

By application of the χ^2 test to the figures in Table I it appears that there is an enhancement of the effect of cold up to 48 hours, but this is less marked later. In these calculations the three experiments with entirely negative results have been omitted, because it is very likely that they were due to a technical error (probably thawing to too high a temperature or leaving too long after thawing before inoculating). In making calculations the readings have been arranged in 15 groups, as shown by the brackets in Table I. The value of χ^2 for the first seven readings is 12.145 with 6 degrees of freedom, which is greater than the value of χ^2 for which P is 6%. The value of χ^2 for the remaining eight readings is 2.444 with 7 degrees of freedom, which is less than the value of χ^2 for which P is 95%. This is compatible with an increase in virulence during the first 48 hours—i.e., during the death of the cells—and a stabilization later. It must be realized that the experiments were not designed for statistical analysis, and no claim is made that the statistical approach offers final

proof of the increase in virulence. It does, however, afford ancillary confirmation, and suggests a rational design for future experiments. The calculations are shown in Table III.

TABLE III

No. of Reading	Total	Observed o	Expected e	$\frac{(o-e)^2}{e}$	
1	18	9	10.62	0.247	$\chi^2 = 12.145$ with 6 degrees of freedom
2	12	5	7.08	0.611	
3	12	2	7.08	3.644	
4	12	8	7.08	0.119	
5	18	6	10.62	2.009	
6	26	23	15.34	3.861	
7	24	19	14.16	1.654	
8	20	13	15.92	0.555	$\chi^2 = 2.444$ with 7 degrees of freedom
9	12	11	9.55	0.220	
10	8	7	6.37	0.062	
11	8	8	6.37	0.417	
12	17	12	13.53	0.173	
13	12	9	9.55	0.031	
14	12	8	9.55	0.251	
15	14	14	11.14	0.735	

My thanks are due to Mr. Richard Gye for these calculations.

II. Experiments in Selective Infectivity

If inbred strains of mice are used tumour tissue can be successfully grafted in both subcutaneous and intra-peritoneal sites. The new tumours are simply clumps of cells of the original mouse growing in the tissues of the new host. If, however, the active form of the Bittner virus has been obtained free from living mouse cells it seems highly probable that only cells of mammary tissue can be infected, and that connective-tissue cells cannot respond to it. Since it is well known that the mammary tissue of the mouse is very widespread, and that sporadic tumours can arise almost anywhere on the surface of the

TABLE IV.—Frozen Carcinoma Inoculated Intraperitoneally

No. of Expt.	Strain	Tumour	Diluent	Time Frozen	Inoculated	Tumours (Intraperitoneal)	Tumours in Abdominal Wall
M60A	MRC/H	63	Dextrose	1 hour	6	1	None
"	"	"	"	3 hours	6	None	None
"	"	"	"	6 "	6	None	1
"	"	"	"	12 "	6	None	None
"	"	"	"	24 "	6	None	None
M53	S/A	Sporadic	None	24 "	4	None	None
M60A	MRC/H	63	Dextrose	2 days	6	None	None
M53	S/A	Sporadic	None	7 "	8	None	None
"	"	"	"	14 "	8	None	None
M57	"	"	"	21 "	4	None	1
"	"	"	"	28 "	4	None	None
"	"	"	"	35 "	4	None	None
"	"	"	"	42 "	4	None	None
M59	MRC/H	63	"	55 "	6	None	1
M58	C3H	Sporadic	Dextrose	118 "	5	2	1
M63	"	"	"	119 "	3	None	2
G8C	R III	"	"	138 "	6	None	None

body, subcutaneous inoculation of active virus can never offer evidence for or against selective infectivity.

On the other hand, sporadic mammary cancer never arises inside the peritoneum, and yet successful intraperitoneal grafts of living tumour tissue can be easily obtained. If, therefore, frozen tumour tissue, which as we have seen readily produces subcutaneous mammary cancers, were inoculated intraperitoneally it should produce tumours if it contained living cells, and should not do so if it contained only active virus. In the experiments shown in Table IV it is obvious (by comparison with Table I) that a qualitative difference in results exists between subcutaneous and intraperitoneal inoculation of refrigerated mammary tumour. The number of failures is too high to be accounted for by any explanation other than the absence of mammary tissue in the site chosen.

The technique of intraperitoneal inoculation is difficult because of the ease with which a few cells from the surface may be carried into the deeper tissues by the needle and also because of the difficulty of avoiding contamination of the abdominal wall and subcutaneous tissues by the material in the needle during its withdrawal. This can be demonstrated in various ways. For example, the inoculum can be mixed with powdered charcoal and its position thus be rendered visible. Small particles of carbon are often found in the abdominal wall at the site of puncture. Again, microscopical examination of subcutaneous and intraperitoneal tumour grafts often reveals the presence of hair follicles which have been forced in by the grafting-needle. A further source of error is a slightly lateral inoculation. Unless the skin and the abdominal wall are pierced exactly in the midline there is always the risk of a mammary tubule being involved in the puncture.

In spite of these obvious sources of error it will be seen that out of 92 inoculations 83 were negative, and of the nine cases in which tumours were obtained six were in the abdominal wall at the site of inoculation and projected as masses of growth on both its superficial and its deep surface. These cases therefore prove nothing, since it is probable that the needle came in contact with a mammary tubule in its passage through the wall. In three of the cases intraperitoneal tumours were found apparently unconnected with any structure, but it is not possible to be certain that they were not originally attached to the parietal peritoneum at the site of needle puncture. In any case a failure to obtain growths in 90% of the intraperitoneal experiments as against a success in 75% of subcutaneous inoculations (where the chances of the virus encountering a mammary tubule are high but not even there 100%) is strongly in favour of selective infectivity of the virus as against cell survival.

Further evidence in this connexion is offered by the behaviour of refrigerated sarcoma tissue on subcutaneous and intraperitoneal inoculation. In both situations the percentage of "takes" is equally high, which suggests a qualitative difference between the carcinoma and the sarcoma virus, the one being adapted to mammary cells only, the other to connective-tissue cells, and therefore able to produce tumours in practically any situation.

Conclusions

The active form of the Bittner factor can be obtained by freezing mammary tumours at -79°C .

The activity of the refrigerated tissue increases rapidly in the first 48 hours and more slowly later.

The refrigerated tissue possesses selective infectivity for mammary tissue, thus differing qualitatively from living tumour cells, which can be grafted in any situation.

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EFFECT OF REPEATED FREEZING AND THAWING ON MOUSE CARCINOMA TISSUE

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From the experiments described in the preceding paper (p. 251) we know that storage of minced mouse carcinoma at -79°C . for long periods does not destroy, but rather enhances, the power of such tissue to start carcinomas afresh when injected into mammary tissue. From those experiments it has been concluded that such "deep freeze" storage kills the cells and renders the Bittner virus available in its active form. The present series of experiments demonstrates that while practically all carcinomas yield active material after one period of freezing (be it for 15 minutes or six months) it is not so easy to produce tumours from material which has been frozen, thawed, and refrozen. In this respect the Bittner milk virus shows itself less thermostable than the sarcoma virus, which will stand repeated freezing and thawing.