Effect of Cold and of Beef Spleen Extract on dbrB Mouse Tumor Cells as Shown by Growth of Transplants into dba Mice and by Cytologic Examination*


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Growth of autografts of mouse tumors previously immersed in aqueous extracts of desiccated embryo skin and of mouse placenta has been retarded and in some instances the grafts have failed to grow (15). Growth of the Bashford adenocarcinoma 63 has been inhibited in mice when the tumor was exposed to aqueous extract of mammary tissue from pregnant rabbits prior to implantation (14). Flexner-Jobling rat carcinoma and mouse sarcoma 180 were stored in the refrigerator for 2 and 5 days respectively with an aqueous extract of rat spleen. The number of takes of the rat carcinoma was reduced upon transplantation, whereas the mouse carcinoma failed to grow (16).

The present paper reports the effects of storage in the refrigerator for 1 to 7 days on tumor cells suspended in an aqueous, alcohol-precipitated beef spleen extract implanted into mice, and a cytologic study at the time of implantation. The investigation was undertaken in an effort to devise an assay method for beef spleen extract which has been useful in the treatment of basal cell epitheliomas in human beings (1, 2).

MATERIALS AND METHODS

The dbrB adenocarcinoma and dba mice were selected for these preliminary tests on the effect of storage in beef spleen extract, since this homologous, non-invasive tumor when implanted into this strain of mice uniformly grows in 100 per cent of the cases and does not undergo spontaneous regression.

Tumors from the donor animals were removed under aseptic conditions and the necrotic portions dissected away. The firm tumor tissue was rubbed through a fine wire mesh and the purée obtained was diluted 3 to 1 by volume with Ringer's solution containing beef spleen extract, and with Ringer's solution alone for the control experiments. The cell suspensions, in stoppered centrifuge tubes, were stored in the refrigerator at $5^\circ \pm 2^\circ \text{C}$.

Isotonic Ringer's solution served as the control storage medium, and the experimental solutions were Ringer's containing beef spleen extract diluted on the basis of 40, 60, 80, 100, and 112 mgm. of solids per ml. of final solution (pH 7.0). Flocculation and gelation of the tumor cell suspensions occurred in concentrations of 80 mgm. per ml. and above. The experimental solution at 78 mgm. per ml. had a tonicity 2.5 times that of isotonic Ringer's solution as determined by the depression of the freezing point so that a control experiment was made with Ringer's solution of 2.5 normal tonicity as a storage medium.

The tumor cell suspension in Ringer's solution had a pH of 6.4 which remained constant for 1 hour, but changed to 6.82 and 6.65 after 1 day and 5 days respectively. Similar suspensions in beef spleen extract diluted in Ringer's solution to 70 mgm. per ml. had pH values of 7.75, 7.30 and 7.29 at the end of 1 hour, 1 day and 5 days respectively.

On the day of inoculation, the cell suspensions were removed from the refrigerator 1 hour before use and gently rotated from time to time. Young dba male mice, 4 to 8 weeks old, were inoculated subcutaneously in the dorsal region with 0.1 ml. of the cell suspension. No perceptible local reaction to the injection occurred, although the tumor derives from its host a rich vascular supply. Once the tumors became palpable (2mm. in diameter) the latent period varying in each experiment, they increased 1 to 2 mm. in each direction each day, the nodules remaining almost free of the skin and underlying tissue. Many of the tumors attained a volume equal to or exceeding that of the host, and the animals eventually died of the tumor. There was no apparent histologic difference in the tumors derived from treated and control cells. A total of 100 mice were injected with the control tumor suspensions, 16 with suspensions stored in...
hypertonic Ringer's solution, and 182 received the suspensions in beef spleen extract of different concentrations. The suspensions were stored at 5° C. in the refrigerator for periods of 1 to 7 days.

Squash preparations from a portion of the tumor cell suspensions as well as from blocks of the tumor tissue were made at the same time as the inoculations of the control and experimental stored tissue. The fixative used was absolute alcohol and glacial acetic acid (3:1) and the preparations were stained with aceto-carmine. Measurements of the nuclear volume were made in 4 preparations of the control and of the experimentally treated tumor cells. Nuclei of 450 to 1200 cu. μ were classified as nuclei of Class II, those of 1200 to 1800 cu. μ as those of Class III according to Biesele, Poyner, and Painter (6). In a few preparations the Feulgen stain was used.

RESULTS

In vivo, control experiments.—Storage of dbrB tumor cell suspensions at 5° C. in isotonic Ringer's solution for as long as 8 days did not greatly reduce the number of tumors developing from the stored cell suspensions upon implantation into test dba mice, 94 tumors appearing in a total of 100 control mice (Fig. 1; Table I). The outstanding effect of storage at 5° C. in isotonic Ringer's solution was progressive prolongation of the normal 6 to 8 day latent period prior to the appearance of palpable tumors (Fig. 2). The values used for the graph are averages of the data presented in Table I. Storage of tumor cells in hypertonic Ringer's solution (equivalent in tonicity to a solution containing 78 mgm. per ml. of spleen extract) for periods of 1, 2, and 8 days reduced the number of tumors developing upon transplantation into mice, and greatly extended the latent period of the tumors which did develop (Figs. 1 and 2; Table I). A seasonal factor may have operated in the 2 and 8 day storage experiment with hypertonic Ringer's solution and in the control tissue, as the experiment was conducted in July and August when the latent period for the appearance of palpable tumors is usually longer than at other seasons of the year.

In vivo, spleen extract experiments.—Storage of tumor cells in 40 mgm. per ml. spleen extract for 1 to 5 days at 5° C. resulted in tumor development in 94 per cent of the 50 test animals and 100 per cent of 30 control animals (Table I). The longer
the period of storage in 40 mgm. per ml. spleen extract the longer the latent period for tumor development, the longest being 18 and 19 days after 4 and 5 days, respectively (Fig. 2; Table I).

Storage of tumor cells in 60 mgm. per ml. of spleen extract for 1, 5, and 7 days resulted in tumor development in 10 of 30 animals as contrasted with 14 tumors in the 15 control animals, or 93 per cent increases. The latent period for the 10 tumors developing after storage for 1 day in 60 mgm. per ml. ranged from 6 to 14 days (average, 8 days). No tumors had developed by the 43rd and 41st day after storage for 5 and 7 days, respectively, in 60 mgm. per ml. spleen extract.

Storage for 1, 2, 3 and 5 days in 80 mgm. per ml. extract resulted in tumor development in 68 per cent of 48 experimental animals, as compared with 100 per cent takes in the 15 control mice. After 1, 2 and 3 days of storage of tumor cells the average latent period for tumor development in 4 groups of mice was 12 (1 day storage), 19 and 20 (2 day storage), and 19 days (3 day storage). In a fifth group of 9 animals receiving tumor cells stored for 2 days in 80 mgm. per ml. the latent period was 8 to 10 days. In this experiment the inoculum spread under the skin and produced multiple palpable nodules 10 days earlier than was the case with the other 2 groups of animals receiving cells stored for the same period of time under the same circumstances (Table I). After 5 days of storage in 80 mgm. per ml. and implantation into 12 mice, no tumors developed within 30 to 43 days of observation (Table I).

Tumor cell suspensions stored for 1 day in 100 mgm. per ml. spleen extract in normal saline produced tumors in 17 of 20 mice after a latent period of 20 to 36 days, whereas cells stored in 100 mgm. per ml. for 2 days had produced no tumors in 20 test mice at the end of 56 days. All 10 of the control animals had tumors within 6 to 14 days (average 11 days).

Of 4 mice receiving tumor cell suspensions stored for 1 day in 112 mgm. per ml. spleen extract, 1 animal developed a tumor after a latent period of 28 days.

Cytology.—Observations on the cytologic effects of storage in the refrigerator on: (a) dividing cells; (b) resting cells; and (c) nuclear volume in isotonic and hypertonic Ringer's solution, and in 40, 60, 80 and 100 mgm. per ml. spleen extract for 24 and 48 hours, and for periods as long as 7 days are successively presented.

Dividing cells. (Storage for 1 day).—Mitotic activity was progressively decreased by storage in isotonic Ringer's solution at 5°C. The proportion of 11 larger polyplorid type of nuclei in 500 cells was unchanged by 24 hours of storage. Mitotic figures were rare after storage for the same period of time in hypertonic Ringer's solution, and while some were normal, usually the chromosomes were clumped at metaphase and at telophase. Storage in spleen extract (40 mgm. per ml.) for 24 hours considerably reduced the number of mitotic figures,
Fig. 3.—Camera lucida drawings of selected nuclei from squash preparations of suspensions of dbvB tumor cells stored at 5° C. for 24 hours in isotonic Ringer's solution and beef spleen extract (60 mgm. per ml.). Alcohol-acetic acid fixative, 3:1, and aceto-carmine stain. Mag. × 2,418.
those present being fairly normal in appearance. In 60 mgm. per ml. spleen extract for 24 hours the number of dividing cells in metaphase was but 6 per cent of the number in the control cells of the same age. The chromosomes were clumped in the few telophase figures observed (Table II). Five of 500 nuclei were of the larger polyploid type (over 1800 cu. μ in volume). Some of the cells containing clumped chromosomes appeared to have been killed in mitosis. After 24 hours of storage in 100 mgm. per ml. spleen extract in normal saline there were very few cells in mitosis and they contained clumped chromosomes.

Storage for 2–7 days.—There were no mitotic figures found in cell suspensions after 48 hours of exposure to hypertonic Ringer’s solution. In cells stored in isotonic Ringer’s solution for as long as 5 days there was during this interval a decrease of 72 per cent in the number of cells in metaphase (Table II). Metaphase chromosomes were clumped into a horseshoe-shaped mass at the end of this period. Occasionally a cell division in telophase was distinguishable but in most instances the chromosomes were clumped into an irregular mass. Rarely, a prophase of an endomitotic type was seen in tumor cells stored in isotonic Ringer’s solution. After as long as 4 days of storage in spleen extract (40 mgm. per ml.) a cell in normal telophase was observed. With 60 mgm. per ml. spleen extract there were very few dividing cells at all times, during the period of storage (Table II), and in these cells the chromosomes appeared abnormal or clumped.

Resting cells. (Storage for 1 day).—In isotonic spleen extract (60 mgm. per ml.). Alcohol-acetic acid fixative, 3:1, and aceto-carmine stain. Mag. X 2,800.
Ringer's solution at 5° C. for 24 hours, the tumor cell nuclei in interphase or resting stage contained usually a large fusion nucleolus 3–5 μ in diameter (Fig. 3), or 2 large nucleoli, and a fine weakly staining reticulum with few and inconspicuous heterochromatric or condensed segments. This type of nuclear morphology replaced that described by Biese (5) as characteristic of malignant cells in mice in which the nuclei had multiple nucleoli and very numerous heterochromatric segments in the reticulum. There were a few smaller tumor cells of the diploid type with conspicuous heterochromatric segments. Both the nuclei and their nucleoli appeared turgid in fixed preparations.

Fragments of resting tumor cells after 24 hours at 5° C. in hypertonc Ringer's solution had a denser cytoplasm than control cells in isotonic Ringer's solution; pointed, pseudopodia-like processes projected from the surface of the cells. The nuclei frequently were distorted and irregular in outline; in some the chromatin reticulum, like that of nuclei stored for the same period of time in isotonic Ringer's solution, was distinct and fine with small heterochromatric knots. In hypertonc Ringer's solution, nucleoli were present in many cells, in some instances were star-shaped, and appeared to be suspended by coarse chromatin threads inside a clear zone in the nucleus. In other cells in hypertonc Ringer's solution, the chromatin reticulum was coarse; the nucleolus when present was small, and it was sometimes absent. In fresh preparations in hypertonc Ringer's solution the nuclear membrane was indistinct. A similar appearance was described by Belar (4) in living spermatocytes of grasshoppers in hypertonc Ringer's solution (5 times that of isotonic Ringer's solution).

Storage of tumor cells in 40 mgm. per ml. spleen extract for 24 hours resulted in little alteration in the chromatin reticulum or in the nucleoli, the latter remaining 3 to 5 μ in diameter as in the control cells. In both 60 and 80 mgm. per ml. spleen extract at 5° C. for 24 hours, the nucleoli were reduced to an average of less than 2 μ in diameter (Fig. 3). In these 2 concentrations of spleen extract, the chromatin reticulum of the tumor cell nuclei was coarser than in the control cells but was regular with very small scattered, darker staining knots in some nuclei; in others the knots were absent. The cytoplasm of these cells appeared denser when stained with aceto-carmine than that of control cells stored in Ringer's solution. In fresh preparations after 24 hours of storage at 5° C. in 65 mgm. per ml., the nuclear membrane was indistinct, an appearance similar to that observed after storage in hypertonc Ringer's solution. After 24 hours at 5° C. in 100 mgm. per ml. spleen extract in normal saline, practically none of the nuclei contained nucleoli or darker staining segments on the fine regular chromatin reticulum. The cells were rounded in form. Longer periods of storage produced little additional change in the cells except for an increase in the number of pyknotic nuclei (Fig. 5).

Storage for 2 to 7 days.—After 4 and 5 days of storage in Ringer's solution, hollow empty nuclei appeared in some of the tumor cells, as well as nuclei with a coarse open reticulum and irregular patches of chromatic material on the nuclear membrane (Fig. 4). After 2 days in hypertonc Ringer's solution (at 5° C.) the reticulum remained fine and there were no normal-appearing, fusion-type nucleoli (Fig. 5). A few star-shaped structures were seen similar to those observed after 1 day of exposure to hypertonc Ringer's solution at 5° C. The contrast in nucleolar form in this and in isotonic Ringer's solution is clearly shown in Fig. 5.
2-Day Storage, 5°C.

Isotonic Ringer's

Hypertonic (2.5) Ringer's

100 mg/ml. Spleen Extract
Tumor cells stored for 4 days in 40 mgm. per ml. of spleen extract resembled those cells stored for 1 day in 60 mgm. per ml. (Fig. 3), the nucleoli being reduced in size and the chromatin somewhat coarse and regular. After 5 days of storage at 5° C. in 60 mgm. per ml. of spleen extract the nuclei contained large clear vacuolar spaces (Fig. 4). The chromatin formed irregular patches on the nuclear membrane connected by a few coarse strands of chromatin. Such nuclei apparently contained no nucleoli. Occasionally there would be a nucleus with a fine chromatin reticulum showing small deeper staining segments. Some of the nuclei were shrunken and wrinkled. The cytoplasm of tumor cells stored for this period of time was more conspicuous than that of cells stored for a similar period in isotonic Ringer's solution. After 7 days of storage at 5° C. in 60 mgm. per ml. spleen extract, the large nuclei were wrinkled and appeared half empty; some appeared pyknotic. Rarely was a mitotic figure observed and in these instances the chromosomes were clumped. A few small round tumor cells with small diploid nuclei having a fine regular chromatin reticulum resembled tumor cells from suspensions stored in 100 mgm. per ml. spleen extract at 5° C. for 2 days (Fig. 5). The cytological picture in general was one of nuclear degeneration and destruction of chromatin.

**Nuclear volume. (Storage for 1, 2 and 5 days).**

The average nuclear volume increased slightly during the first 2 days of storage in isotonic Ringer's solution over that of unstored nuclei. Of 50 nuclei measured at the end of 24 hours of storage, 48 per cent were over 1,200 cu. μ of the larger Class III size of nucleus, according to the classification of Biesele, Poyner, and Painter (6); after 5 days, 40 per cent were of this type. The rest of the nuclei in both cases were under 1,200 cu. μ in volume, or Biesele's Class II diploid type. After 1 day in 60 mgm. per ml. spleen extract at 5° C., 40 per cent of the measured nuclei were of the Class III size, and after 5 days 23 per cent remained in this classification, indicating that the 60 mgm. per ml. spleen extract acting for 24 hours at 5° C. had had the same effect on nuclear volume as 5 days in isotonic Ringer's solution at 5° C. The average nuclear volume of 50 cells stored for 24 to 48 hours in 100 mgm. per ml. spleen extract in normal saline at 5° C. was less than that of control cells in isotonic Ringer's solution.

**DISCUSSION**

The observed effects of cold on dbrB tumor cells in isotonic Ringer's solution are in agreement with the report of Heilbrun (11) that cold prevented spindle formation in sea urchin eggs and that normal mitosis was resumed when the chilled cells became warm again. In contrast to the findings of Barber and Callan (3) that metaphase configurations increased 260 per cent in newt larvae during an 8 day storage period at 3° C., we observed but few nuclei in metaphase during storage of dbrB tumor cell suspensions in isotonic Ringer's solution at 5° C. for as long as 7 days.

Many of the resting nuclei of the stored tumor cells resembled the non-proliferating malignant or Type B cells described by Koller (12, 13). The nucleoli of variable size in these non-proliferating cells consist of histone-protein and ribose nucleic acid with very little of the desoxyribose nucleic acid (12) which latter predominates in the small nucleoli of proliferating cells; these 2 nucleic acids are convertible one to the other in a reversible reaction in the normal mitotic cycle (8, 9). It is probable in our experiments that the resting type of nucleus in the tumor suspensions was reconverted into the proliferating type of nucleus upon injection into mice, since the takes were 100 per cent in nearly every control experiment. Among the smaller diploid nuclei in tumor cells stored in isotonic Ringer's solution for as long as 7 days, there were a few resembling those of the Type A proliferating cells described by Koller (12, 13) as having small Feulgen-positive nucleoli and conspicuous heterochromatic segments on the nuclear reticulum.

When dbrB tumor cells were stored in beef spleen extract at 5° C., the immediate elimination of almost all mitotic activity, the reduction of nucleolar size, and the presence of a coarse reticulum lacking darker staining segments, can be correlated with the number of tumors developing in the injected mice. When the tumor takes were 100 per cent with cells stored for 1 day in 60 mgm. per ml. spleen extract and for 4 days with 40 mgm. per ml. spleen extract, the nuclei were cytologically similar. When no tumors developed following injection of cells stored for 5 days with 60 mgm. per ml. spleen extract, the cytologic picture was one of destruction of nuclear material as manifested by empty and semi-empty nuclear membranes and by pyknosis; after 5 days of storage in isotonic Ringer's solution, tumor cell suspensions gave rise to tumors in 100 per cent of the animals although a few of the cells cytologically showed a decrease in chromatin content of the nuclei (Fig. 4). When tumors developed in all of the animals receiving
cell suspensions stored for 1 day at 5° C. in either hypertonic Ringer's solution or in 100 mgm. per ml. spleen extract, the chromatic reticulum in both instances was a fine regular network; the nucleoli persisted in those cells stored in Ringer's solution but had disappeared from most of the cells in the presence of the extract. Although there was little further cytologic change in these cells after 48 hours (Fig. 5), tumors developed in but 1 of 6 mice receiving suspensions stored in the hypertonic Ringer's solution, and in none of the 20 mice receiving those stored in 100 mgm. per ml. spleen extract.

A correlation of the cytologic characteristics of tumor cells stored in spleen extract at 5° C. with the results obtained in vivo, showed that the reduction in the number of tumors developing may be due to: (a) an immediate destruction of some cells; and (b) an accelerated degeneration of the nucleus with a deterioration of its contents.

The action of the spleen extract may be due in part to its hypertonicity since in its higher concentrations the large polyploid cells were damaged first and mitosis was inhibited almost immediately. Belar (4) observed that in living grasshopper spermatocytes exposed to hypertonic Ringer's solution, the cytoplasm was first affected, the spindle next, and finally the chromosomes. The prolonged latent period before tumors became palpable, in those instances in which tumors developed the latent period was prolonged over and above that for tumors from suspensions stored for the same period of time in isotonic Ringer's solution.

Although the nucleolus tended to persist in nuclei of cells in hypertonic Ringer's solution, and to disappear in those in spleen extract, a toxic effect of the extract on the malignant cells cannot be eliminated as a possible factor in the reduction of tumor takes from cells stored in spleen extract. Dustin (10) observed in the nuclei of intestinal epithelium of mice that in vivo the nucleolus was apparently more resistant than other cellular structures to poisons such as hydroquinone and the carbamates, which he classifies as radiomimetic poisons, and which seem to act on the enzyme systems. Nuclei of potentially dividing cells are most sensitive to the action of poisons just prior to prophase. The appearance of the nuclei as described by Dustin (10) during degeneration and pyknosis is similar to that of dbrB tumor cell nuclei after 5 days of storage with 60 mgm. per ml. spleen extract. Dustin (10) described the effects of another group of poisons, i.e., mercurial compounds, arsenite and colchicine, on dividing cells in vivo as preventing spindle formation with a consequent accumulation of metaphase figures. This effect was not observed in vitro in dbrB tumor cells exposed to spleen extract, nor when 0.5 ml. of 260 mgm. per ml. spleen extract was injected into a small growing tumor 2 days before its excision; mitotic divisions were found with high frequency in these tumor cells. Neither resistance to nor regression of the dbrB tumors has been produced with the beef spleen extract. Cameron and her associates (7) have shown that the response of mouse tumor cells to organic poisons is not necessarily similar to that of human malignant cells.

Storage of mouse tumor cells in beef spleen extract at 5° C. has proved unsatisfactory as a method for assaying the potency of the spleen extract for clinical investigations.

SUMMARY

Injection of dbrB tumor cell suspensions after storage at 5° C. for 1 to 7 days in different concentrations of an aqueous extract of beef spleen resulted in a reduced number of tumors developing, this reduction being related to the dilution of the extract and the period of storage. In those instances in which tumors developed the latent period was prolonged over and above that for tumors from suspensions stored for the same period of time in isotonic Ringer's solution.

Cytologic examination revealed that in the presence of the spleen extract at 5° C. cell division disappeared, the nucleoli and heterochromatic segments were reduced in size, the number of vacuolated nuclei was increased, as well as the destruction of chromatic material leading to pyknosis and to hollow nuclear membranes.

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Effect of Cold and of Beef Spleen Extract on dbrB Mouse Tumor Cells as Shown by Growth of Transplants into dba Mice and by Cytologic Examination

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