Comparative Effects of Chemotherapeutic Agents on Brown-Pearce Tumor and Normal Rabbit Testis *in vitro*.* (20689)

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(Introduced by Alfred Gellhorn.)

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Evidence has recently been cited(1) that the effectiveness of the guanine analog, 8-azaguanine, as a tissue inhibitor is related to the rate at which the tissue is able to deaminate this compound to its completely inactive derivative 8-azaxanthine. Normal and neoplastic tissues with a high deaminase activity were shown not to be susceptible to 8-azaguanine, whereas tumors in which the deaminase activity was low were inhibited. Chromatographic tests of feeding fluids from human glioblastomas cultivated *in vitro* have indicated this further, these susceptible tumors being almost entirely lacking in deaminating capacity(2). By this method the feeding fluids from tissue cultures have provided a more sensitive test of deamination than tissue homogenates. Since the Brown-Pearce rabbit carcinoma is known to be susceptible to 8-azaguanine *in vivo*(3,4) and since homogenates of this tumor as well as of normal rabbit testis have a negligible deaminase activity(1), it seemed desirable to study the effect of this agent on these tissues *in vitro*. Chromatographic tests of the feeding fluids were made to determine the azaguanine-deaminating capacity of the tissues. For comparison, the effects of the structural analogue 8-azaxanthine, and of benzimidazole(5), and 1,4-dimethanesulfonyloxybutane(6), an unrelated aliphatic carcinostatic agent, were also investigated.

Material and methods. The Brown-Pearce tumors used for culture were carried in the anterior chamber of the rabbit's eye. They were grown *in vitro* by the Maximow double coverslip method using a feeding medium of 3 parts human placental serum, 1 part beef serum ultrafiltrate and 1 part chick embryo extract. The tissue was cultured for one week during which the feeding solution was changed 3 times and the explants patched with chicken plasma once. At the end of this period the experimental solutions were added to the feeding media in the desired concentrations. For each test approximately 50 explants from 3 different tumors were used. The testicular tissue used for cultivation was obtained from healthy 2-3-month-old rabbits. It was grown *in vitro* in the same manner as the tumor tissue and was cultured for one week before the addition of the desired agent. Approximately 50 explants from 3 different rabbits were used for each test. A 0.1 M stock solution of 8-azaguanine was prepared by heating the solid in one-third the final volume of 0.5 N NaOH until a clear solution was obtained. This was then adjusted to pH 8 with 1 N HCl and made up to volume. It was applied to the tumor cultures in final concentrations of 0.0003 M, 0.0008 M, and 0.0016 M, and to the testis cultures in final concentrations of 0.0008 M and 0.0016 M. A control solution containing only NaOH in the above concentration was included in each experiment. The 8-azaxanthine solution was prepared in the above manner and applied to the tumor in a final concentration of 0.0016 M. Benzimidazole was prepared in a similar manner using 0.1 N NaOH instead of 0.5 N NaOH and was employed in final concentrations of 0.0016 M and 0.005 M. An 0.1 M stock solution of 1,4-dimethanesulfonyloxybutane was prepared by dissolving the solid in one-third the desired volume of propylene glycol and diluting to volume. This solution was applied to the tumor in final concentrations of 0.01 M, 0.005 M and 0.0016 M. A control containing propylene glycol alone was used in this case.

For permanent record and detailed study the cultures were fixed in Helly's fluid and stained with Harris' haematoxylin at the conclusion of the experiments. For the chromato-

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TABLE I. Effects of Chemotherapeutic Agents on Brown-Pearce Tumor and Normal Testicle *In Vitro*.

Effect	Day of occurrence: .0016 M 8-Azaguanine — Brown-Pearce tumor	.0016 M 8-Azaguanine — Normal testicle	.005 M Benzimidazole — Brown-Pearce tumor
Increased cytoplasmic granulation	1–2	1–2	1–2
Nuclear membrane more distinct	—	—	1–2
Elongate cells clubbed	—	1–2	2–3
Clumping	1–2	—	—
Proliferation stopped	2–3	2–3	3–4
Binucleate or multinucleate cells formed	ca. 2 mostly binucleate	—	ca. 5 multinucleate
Migration slowed	ca. 2	—	4–5
Round-cell shrinkage, pyknosis	2–3	—	blebbing 3–4
Vacuolization	—	—	ca. 5
Nuclear disintegration	ca. 3	3	5–7
Cell disintegration	4–5	4–5	ca. 7
Migration stopped	5	5	9–10

graphic tests the tumor or testicular tissue was grown in roller tubes. Each tube contained 5 explants which were fed a medium consisting of 3 parts balanced saline, 3 parts human placental serum, 3 parts beef serum ultrafiltrate and 1 part chick embryo extract. The tissue was cultivated for one week after which 8-azaguanine was added to the feeding fluid so as to produce a final concentration of 0.005 M. A control containing NaOH alone was used. Collection of the feeding fluids was made after 24 hours. The determinations were made in the same manner as those of the human glioblastomas(2).

Results. The Brown-Pearce tumor proliferates fairly rapidly *in vitro* and is characterized by two types of cells—one of elongate fibrocytic appearance and the other round and of fairly uniform size. The cells are mononuclear for the most part and tend to grow in sheets from the explant. The morphological characteristics of this tumor in tissue culture have been more fully described by von Möllendorff(7) and by Favorite and Cheever(8).

Of the 4 compounds under investigation, 2 failed to exert any demonstrable effect on the Brown-Pearce tumor *in vitro*. 8-Azaxanthine, although applied to the tumor at a concentration comparable to a consistently lethal level of 8-azaguanine (0.0016 M), had no cytological or inhibitory effect during the 10-day exposure period. 1,4-Dimethanesulfonyloxybutane was inactive at concentrations of 0.01, 0.005, and 0.0016 M. Table I summarizes the results obtained with the 2 active agents. 8-Azaguanine in general caused the round cells to shrink and become more granular. The cells tended to stick in clumps instead of migrating in the normally diffuse manner. Inhibition of cell proliferation occurred, followed by an increase in binuclear forms, later by nuclear and cell disintegration and finally by total inhibition of cell migration. The rate at which these effects were exerted varied directly with the concentration—the 0.0016 M solution was lethal in 5 days and the 0.0008 in 10 days. The 0.0003 M solution failed to cause a complete inhibition of cell proliferation or migration within the 10-day test period. The number of binucleate cells also seemed to be correlated with the concentration—the 0.0016 M producing the most and 0.0003 M the least. In contrast to the round cells of the tumor, the fibrocytic type of cell was not affected at any of these concentrations.

Benzimidazole when applied in a 0.0016 M solution did not affect the tumor in any way, but at 0.005 M inhibited proliferation in 3 to 4 days and migration in 10 days. This lethal effect was accompanied by a marked increase in the number of multinucleate cells. The cells also were shrunken and had more distinct nuclear membranes and a more granular and vacuolar cytoplasm than did the controls, and they also developed blebs or blisters. These changes were accompanied by nuclear and finally by cellular disintegration. Benzimidazole also affected the *fibrocytic* type of cell,

causing it to become more blunt and rounded than normal. In general, however, this agent was significantly less inhibitory than 8-azaguanine.

Normal testicular tissue proliferates rapidly *in vitro* forming large colonies of fibroblast-like cells among which occasional round spermatogonial cells are found. When treated with 8-azaguanine complete inhibition was obtained in 10 days with the 0.0008 M and in 5 days with the 0.0016 M. In both cases, the cells became more granular and less elongate and branched than normal. The nucleus tended to round up and become more conspicuous. The nucleoli were more darkly staining than those of the controls. This was followed by nuclear and cell disintegration. Spermatogonial cell formation was also completely inhibited.

The chromatographic determination of azaguanine deaminase activity of the Brown-Pearce tumor and normal testicle demonstrated that a very large portion of the 8-azaguanine added in the feeding fluids (85-88%) remained unchanged after a 24-hour incubation period, indicating that the deaminase activity of these two 8-azaguanine-susceptible tissues was very low.

Discussion. Two main points of interest emerge from these comparative experiments. The effectiveness of 8-azaguanine and the complete inactivity of 8-azaxanthine are entirely consistent with the pattern demonstrated in a variety of tumors *in vivo*(1) and in the human glioblastomas *in vitro*(2). The inhibitory effect of 8-azaguanine on tissue cultures of this tumor appears to be correlated with the inability of the tissue to deaminate the carcinostatic agent to the inactive derivative, 8-azaxanthine. The negligible azaguanine deaminase activity of both the tumor and normal testicle *in vitro*, demonstrated by paper chromatography, reinforces this correlation.

Tissue cultures of this rabbit tumor and of rabbit testis are much more susceptible to 8-azaguanine than those of the human glioblastomas studied in earlier experiments(2); comparable effects were obtained at concentrations of 0.0008 M in the first two tissues and 0.005 M in the latter.

A comparison of the effects of these 4 agents on the Brown-Pearce tumor *in vivo* and *in vitro* points up significant discrepancies, as well as similarities, between these biological systems. On the one hand, the action of 8-azaguanine and 8-azaxanthine on this tumor is the same in the anterior chamber of the eye and in tissue culture: the former is strongly inhibitory in both systems and the latter has no effect. On the other hand, there is a wide divergence between the results *in vivo* and *in vitro* in the experiments with the other two agents. Benzimidazole, which does not cause any appreciable inhibition of the Brown-Pearce tumor in anterior chamber transplants (9), inhibits the growth of this tumor in tissue culture; 1,4-dimethanesulfonyloxybutane, which has been shown to have an inhibitory action *in vivo*(10), fails to affect the tumor *in vitro* in any way, even at levels 12 times as great as the least effective concentration of 8-azaguanine. These discrepancies invite speculation, but no established facts can be adduced at the present time to explain them.

Summary. 1. The effects of 8-azaguanine, 8-azaxanthine, benzimidazole, and 1,4-dimethanesulfonyloxybutane on the Brown-Pearce tumor *in vitro* were investigated. 8-Azaguanine proved lethal in 0.0016 M concentration in 5 days and in 0.0008 M concentration in 10 days, and benzimidazole was lethal in 0.005 M concentration in 10 days. 8-Azaxanthine and 1,4-dimethanesulfonyloxybutane proved ineffective in all concentrations used. 2. The comparative effects of 8-azaguanine on the Brown-Pearce tumor, which does not exhibit azaguanine deaminase activity, and on the normal rabbit testis, which also lacks this deaminase, were studied; and in both instances complete inhibition was obtained in the same time and at the same concentration. Paper chromatography tests on the feeding fluids of these 2 tissues also showed them to have negligible deaminase activity. 3. No reason is yet known for the disagreement, in the case of this rabbit tumor, between the results *in vivo* and those *in vitro* as regards inhibition by benzimidazole and by 1,4-dimethanesulfonyloxybutane (Haddow's GT 41); for 8-azaguanine, however, the results are consistent in

animal and tissue culture.

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Effect of Aliphatic Oxime and Isatin Thiosemicarbazones on Vaccinia Infection in the Mouse and in the Rabbit.* (20690)

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Mice inoculated intracerebrally with vaccinia virus and treated with certain thiosemicarbazones containing either benzene or heterocyclic groups are protected against the virus (1-3). The concentrations of virus in the brains of mice treated with isatin thiosemicarbazone (T.S.) and sacrificed 5 days after initiation of infection was found not to be significantly different from those present in untreated animals(2). Further investigation of the mode of action of thiosemicarbazones in vaccinia infection has demonstrated 1) that serial passage of the virus in treated mice results in a gradual decrease in the amount of virus demonstrable in the brain; 2) that isatin T. S. fails to protect rabbits against vaccinia virus inoculated by the cerebral route; and 3) that aliphatic oxime derivatives of thiosemicarbazone may possess the capacity to protect mice against vaccinia virus. Results obtained from these experiments will be presented in this report.

Methods and materials. The International Health Board (IHD) and the Williamsport strains of virus were used(1,2). Infected mouse brain (IHD strain) or rabbit testicular tissue (Williamsport strain) was ground in a mortar and diluted with Locke's solution containing 5% normal rabbit serum. Harlan Swiss female mice weighing from 15 to 20 g were anesthetized with nembutal and inoculated intracerebrally with appropriate viral dilutions. Rabbits weighing approximately 2 kg, each, were inoculated either intracerebrally or intracisternally. Test materials were fed in a casein-sucrose diet or injected intraperitoneally as a suspension in sterile distilled water.

Results. Two experiments were caried out to determine the effect of serial passage of the Williamsport strain of virus in mice treated intraperitoneally with isatin T.S. (Table I). Animals were treated on the day of viral inoculation and on 2 succeeding days and sacrificed on the 5th day. Brains were removed from 6 mice after each transfer; those found free of bacterial contaminants were pooled and appropriate dilutions prepared. Mice were inoculated for transfer purposes and the viral content of the pool determined by duplicate intradermal titrations in 2 rabbits.

In Exp. 1, mice were treated with 5 mg doses of the compound and passages were

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