In 1926 Murphy (13) reported successful cultivation of the Jensen rat sarcoma on the chorio-allantois of the chick embryo and subsequent transplantation of the "egg" tumors to rats. The tumors were carried for 4 serial passages in eggs before being lost. The survival time of the tumor cells was tested by inoculation into adult or newly hatched chickens. It was found that, while the cells of tumors from rats would survive for 3 days under these conditions, those of the tumors grown in eggs disappeared completely within 24 hours. Because of this fact, Murphy concluded that the cells of the Jensen sarcoma appeared to have become less resistant during their growth in the egg.

Taylor and his co-workers (18, 20) recently described the successful cultivation of mouse tumors in the yolk sac of the chick embryo. Taylor later reported the presence of a cell-free agent in the yolk surrounding such tumors that caused the rapid production of tumors when inoculated into mice (17).

One of us (11), using the technic described by Taylor and his associates, observed that a mammary carcinoma from a mouse of the inbred C3H strain would grow in the chick embryo, and that these tumors could be transplanted to mice of the parental stock.

**MATERIAL AND METHODS**

Spontaneous mammary carcinomas originating in mice of the C3H or the A strain were used in this study. Histologic sections showed them to be highly undifferentiated tumors typical of those originating in these strains of mice.

The method of yolk-sac cultivation was essentially that described by Taylor and his group (18). In the present study, however, the inoculum was prepared under a hood by grinding the tumor in a mortar together with a physiologic solution of sodium chloride. In most cases the tumor material was weighed and a 40 per cent suspension prepared for inoculation, though occasionally the material was not weighed and a 40 per cent suspension was approximated. Two-tenths cubic centimeter of this suspension was then inoculated into the yolk sac of the 5-day chick embryo. Previous experiments had shown that considerable variation in the size of the inoculum made no difference in the growth of the tumors. All the inoculations were performed by the same worker. With a few exceptions, the inoculated eggs were candled daily to test for viability.

After incubation of the inoculated eggs the surface of the shell was sterilized by immersion in a solution of 2 per cent iodine in 70 per cent alcohol for 2 minutes, followed by 70 per cent alcohol for 2 minutes. The eggs were then drained, crushed between the halves of a sterile Petri dish, examined with sterile instruments, and the tumors in the yolk sac were measured and ground in saline solution for further passage. The average number of eggs inoculated at each passage was 10. All eggs used in this study were from a private flock of white leghorn chickens.

**Observations During Serial Yolk-Sac Passage**

The C3H tumor was implanted in the yolk sac on May 31, 1943, after 13 passages in mice of the parent strain or their F1 hybrids. During the early serial yolk-sac passages of the tumor transfers were made at 12-day intervals. After 8 passages the majority of the embryos began to die before the 12th day after inoculation, and it was necessary to make transfers at shorter intervals in order to maintain the tumor. With continued passage the embryos died at shorter and shorter periods after inoculation, and on a number of occasions during the later of the 20 passages most of the embryos died the night before transfer was contemplated, leaving only 1 or 2 alive from which the tumor could be transferred. The consecutive intervals of transfer for the first 19 of the yolk-sac passages were 12, 12, 12, 12, 12, 12, 12, 12, 12, 11, 12, 11, 10, 11,
9, 9, 9, 9, 8, 8, and 8 days respectively. In the 20th passage the 6 eggs that were to have been opened on the eighth day after inoculation died on the night of the seventh day, December 20, 1943, and the egg passage tumor was lost.

The tendency of the embryos to die at progressively shorter intervals is shown by the percentage surviving over an arbitrarily chosen period. The percentage of embryos surviving through the ninth day was computed for each of the first 16 egg passages. The average percentage surviving in egg passages 1 through 4, 5 through 8, 9 through 12, and 13 through 16 was 63, 56, 49, and 28 respectively. Since transfers after the 16th passage were made at intervals of 8 days the number of survivors cannot be compared with the foregoing series. The average proportion of survivors over a period of 8 days for the final 4 passages was, however, 29 per cent.

The increasing mortality rate of the embryos on continued egg passage of the tumor was not associated with an acceleration of its growth rate. During the early passages at 12-day intervals the tumors were relatively large, commonly measuring 9 to 17 mm. in diameter and weighing 1 to 3 gm. With passage at shorter intervals the tumors were correspondingly smaller. The eggs in the later passages of the series that were opened after 8 or 9 days of incubation yielded only relatively small tumor nodules, 3 to 4 mm. in diameter, around the edge of the umbilicus of the yolk sac. The increasing mortality rate of the embryos in the later passages was thus not related to the size of the tumors.

Cultures of the yolk were made in approximately half of the cases in which the embryos died spontaneously. In only 3 was there evidence of bacterial contamination, and in each case this contamination was due to micrococci. It is evident that bacterial contamination did not explain the spontaneous deaths of the inoculated embryos. The tumor material used for transfer in the series was found in every instance to be bacteriologically sterile by cultures in dextrose-brain broth and blood agar.

Repeated efforts to carry a mammary carcinoma originating in the A strain by serial yolk-sac passage were less successful than the attempts to carry the C3H tumor. The primary inoculation with mouse tumor suspension yielded relatively large growths after 12 days of incubation, but the second or third yolk-sac transfer resulted in death of the embryos after 3 to 6 days. With this tumor it appeared that the tendency to produce a lethal effect upon the embryo was attained much more rapidly on yolk-sac passage than in the case of the C3H carcinoma.

Growth of the C3H Yolk-Sac Passage Tumor in Foreign Strains of Mice

The susceptibility of inbred strains of mice was tested to grafts of two "egg" tumors that had been transplanted into mice after the fifth and the 11th serial passage in eggs. These tumors are designated as H5 and H11 respectively.

The tumors were carried in mice of the C3H strain, C3H F1 hybrids, and C3H back-cross mice. In addition, mice of the following strains were tested: A, C57 black or B, C albino, and sublines 212 and 12 of the D or dilute brown stocks.

Both tumors grew rapidly in C3H and their hybrid animals and were transplanted every 10 to 14 days, usually by the trochar method.

The H5 tumor was inoculated into 120 C3H mice or their hybrids and 11 animals of the same groups were used to continue the H11 tumors. The tumors grew progressively in all animals. There was no apparent difference in the rate of growth of the two, although they were not transplanted simultaneously in many animals to test this fact. The majority of the mice did not have the active milk agent.

On August 18, 1943, 14 mice of the inbred A strain were inoculated subcutaneously with grafts of the C3H tumor, H5. None of the animals gave palpable nodules. These mice were reinoculated on August 30, 1943, with the same tumor and again the results were negative.

Fifty mice of the C57 black, C albino, A albino, and sublines 212 and 12 of the dilute brown stocks were inoculated with tumor H11 on November 10, 1943, and the surviving animals were reinoculated with the same tumor on December 11, 1943. In 47 (94 per cent) tumors developed, ranging from 0.5 to 5 cm. in diameter. Three of the 47 mice, all members of subline 12 of the dilute brown stock, died, presumably from their growths. All the other tumors had regressed within 4 weeks. None of the mice that had tumors after the initial inoculation showed growths after reinoculation. Of the 3 mice that were negative after the initial inoculation, 2 showed small tumors after the second inoculation. These growths regressed within a few weeks.

Filtrates (Berkefeld and Seitz) of the H5 tumor were injected subcutaneously into males of the highly cancerous C3H strain. Tumors did not result. Filtrates of the H11 tumor were not tested.

Attempts to Find a Cell-Free, Tumor-Inducing Agent in Tumor-Bearing Eggs

Several attempts were made to find a cell-free, tumor-inducing agent in material from tumor-bearing

- Five mice of subline 12 were inoculated. Four mice of subline 212 had temporary masses after the initial inoculation.
eggs. In each instance material from eggs bearing relatively large tumors, which weighed from 1 to 3 gm., was used for study. In every case the material was taken from eggs containing living embryos, and was handled as rapidly as possible to prevent inactivation of any tumor-inducing agent that might be present. Tests for such an agent were performed by the subcutaneous injection of 0.8 cc. of the material into the mammary region of adult female mice of the parent strain or parent strain back-cross mice.

Experiments with the C3H tumor.—When the untreated yolk surrounding large tumors was injected subcutaneously into mice, tumors commonly developed in 10 to 30 days. Five different specimens of yolk were inoculated into a total of 8 mice, and tumor growth resulted in 6. Histologic sections of the tumors showed them to have a structure similar to that of the original mouse tumor. It appeared probable that there were living tumor cells in the yolk surrounding the tumors.

Several yolks, together with an equal volume of saline solution, were passed through a Berkefeld N filter and inoculated into 2 mice. No tumors appeared during 112 days of observation. Even when yolk material was diluted 4 times with saline solution the pressures necessary to force the mixture through Berkefeld N filters, as measured by a mercury manometer, were so great as to make the filtration unreliable. Because of this fact attempts to obtain a cell-free agent by filtration were not continued.

On 2 occasions 3 yolks were pooled and rapidly frozen and thawed 3 times by alternate immersion in a mixture of carbon dioxide snow and alcohol, and in water at room temperature. This material was injected at once into mice, 3 for each of the 2 specimens. After 42 days there was no evidence of tumor growth.

Another specimen containing several yolks was shaken with cold ether and quickly centrifuged. The supernatant ether was discarded and part of the remaining material was injected into 2 mice. After 62 days no evidence of tumor growth was present.

In 2 instances the blood and allantoic fluid from several embryos bearing large tumors in the yolk sacs were centrifuged at 3,000 revolutions per minute for 5 minutes in an angle centrifuge. The sediment from each specimen was injected into the mammary region of 2 mice. At the end of 33 days the 4 mice did not show any evidence of tumor growth.

Experiments with the A tumor.—Three yolks and yolk sacs were emulsified in a mortar together with 2 volumes of saline solution, filtered through paper by suction, and frozen in a mixture of carbon dioxide snow and alcohol. The thawed material was injected into 3 mice. This experiment was repeated on another occasion. After 30 days none of the 6 mice showed evidence of tumor growth.

Three different specimens, each containing several yolks and yolk sacs, were frozen and thawed rapidly 3 times and injected into a total of 8 mice. After 60 days of observation there was no evidence of tumor growth.

COMMENT

The reason for the increasingly lethal effect on the chick embryo of the implanted C3H strain tumor during serial transfer in the yolk sac is not clear. The possibility that a virus accompanied the tumor and gradually increased in virulence with passage may be considered. Taylor and his co-workers (19) have described a depression of the hemoglobin level in embryonic chicks bearing an implanted tumor in the yolk sac, the severity of the depressant action being in direct relation to the size of the growth. In the present experiments the lethal effect on the embryo was not in relation to the size of the tumor. A change of susceptibility in the embryos could explain the increasing mortality rate in the later yolk passages of the tumor, but this hypothesis is not probable, for during the entire period of transfer of the egg-passage tumor other growths were being inoculated successfully from mice into eggs and were growing to a large size.

The successful transplantation of tumors into mice is usually dependent upon the relation of the genetic constitution of the tumor grafted to that of the host inoculated (12). Each transplanted tumor, even each one of multiple spontaneous tumors from a single host (4, 5, 8, 16), has been found to have a definite genetic constitution, and only animals that have the same growth factors will respond by allowing the tumors to grow progressively.

Mammary neoplasms have been observed to "mutate" during the process of transplantation (4, 8, 14, 15), that is, they become less specific, and require a smaller number of growth factors for successful transplantation. The growth rate of the mutant tumors was usually greater than before mutation, but their histologic appearance was not necessarily changed.

It has been unusual to find tumors developing in mice of inbred strains that would grow progressively in those of unrelated homozygous strains (6), but after mutational changes Strong (15) found one that would grow in all mice and Cloudman (8-10) described 2 transplantable mammary tumors that would grow in mice other than those of the parental strain. It was not stated whether these tumors would grow in this manner when they were first transplanted or whether the specificity of the cells had changed during propagation.
Attempts to immunize mice of inbred strains against their own tumors have usually been unsuccessful (3, 7). On the other hand, it has been possible to induce immunity in mice of inbred strains to a few tumors that had developed in mice of unknown ancestry but would grow progressively in these animals (for literature see 1, 2, 3, 7). In these experiments it was determined that the genetic constitution of the inoculated host played an important role in the development of immunity. Likewise, the site of subcutaneous inoculation had to be considered. That is, if the grafts were placed in positions or sites where the blood supply was not as abundant as in others they would grow more slowly; as a result, more tumors would regress and the host would be immune against other grafts of the same or other tumors (2, 7). In one study (7) the temporary growth of the tumor was not a prerequisite for immunity.

Barrett (3) found that immunity to tumor 15091a (8-10), which developed in a mouse of the A strain, might be induced in unrelated strains by the injection of defibrinated blood from mice of other stocks, whereas homologous blood gave insignificant differences. Thus the degree of resistance depended upon the genetic relation of the host, the tumor, and the donor. Immunity could not be produced in mice of the A strain.

In the present experiment it seemed probable that the genetic constitution of a mammary carcinoma from a mouse changed during serial passage in chick embryos. Previous to the "mutation" the tumor from eggs would grow progressively when transplanted into mice of the inbred strain in which it developed, but would not give palpable nodules in mice of another inbred strain. After the change of specificity, between the sixth and 11th passage in eggs, grafts would show temporary growth in mice of several unrelated inbred strains. After spontaneous regression of their tumors the mice were resistant to further inoculation of the same growth. Mice of the strain in which the tumor developed grew the tumor progressively and few of the animals survived for longer than 3 weeks after inoculation.

The several attempts to obtain a cell-free agent, capable of inducing tumors in mice, with material from tumor-bearing eggs resulted in failure. Many of the known viruses would have withstood the freezing and thawing method used to destroy the cells in these experiments, but since the number of animals used in these attempts was small no definite conclusions can be drawn.

SUMMARY

A mammary carcinoma of the mouse has been cultivated in the yolk sac of the developing chick embryo for 20 serial transfers. With yolk-sac passage of the tumor the embryos died at progressively shorter intervals. Death of the embryos was not related to the size of the tumor.

Transplantation of the tumor into mice after serial passage in chick embryos gave data that suggested changes in the genetic constitution of its cells while they were being propagated in the eggs.

Previous to the change mice of an unrelated strain were resistant to inoculation, but after the "mutation" mice of several stocks showed temporary growth of the tumor. After regression of the transplants mice of the unrelated strains were immune to reinoculation.

Whereas none of the mice of the strain in which the tumor developed survived inoculation, only a few mice of one unrelated strain showed progressive growth of the grafts.

A limited number of attempts to find a cell-free agent in material from tumor-bearing eggs that would produce tumors in mice resulted in failure.

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