A Tissue-specific Primary Response to Sarcoma 37 in the Chick Embryo

RONALD C. FRASER

(Department of Zoology, University of Tennessee, Knoxville, Tenn.)

Most work on the growth of heterologous tumors has made use of embryonic hosts in which a defense mechanism has not yet been established. Koprowski and associates (8) have successfully transplanted a rat hepatoma in ascitic form into mice, after the inoculated cells had been pretreated by serial passages through mice embryos. Numerous investigators have used the chick embryo as a host for various mammalian tumors (2, 9, 11, 14; see 7 for a review on this topic). The target tissues and routes of inoculations in the avian embryo have varied, namely: (a) the chorioallantoic membrane (7, 11, 14), (b) the intravenous route, via the allantoic vessels (1, 3), (c) the intra-embryonic route (2, 6, 9), (d) the yolk sac (5, 15), and (e) an in vitro mixture of tissues (10, 16, 17).

In time during its development the chick embryo develops a defense against the foreign cells and destroys them. The time in which this resistance to heterografts develops has been reported most frequently as 17–18 days of incubation (4, 5, 14), although there are some indications that serum antibodies (8) and complement (12) are not detectable until after the time of hatching. It has also been reported that Sarcoma 37 cells are destroyed by macrophages present in chick tissues cultured in vitro (10).

To evaluate the role played by circulating blood and isolated tissue in the initial response of the chick embryo to mouse tumor cells, heterografts were made to the chick chorioallantoic membrane (CAM) and to isolated fragments of tissue cultured in vitro. The CAM was chosen as an environment rich in blood, yet containing relatively little tissue substratum and located quite apart from the embryo proper. The source of blood-deficient tissue consisted of fragments of limb musculature, or entire limbs of younger embryos, grown in organ culture on a medium appropriate to chick tissues. The conclusion drawn from observations on the mouse Sarcoma 37 cultured in association with chick tissue in this manner is that, even before an immune mechanism has had time to become established in older embryos, the muscle tissue environment, but not that of the CAM, is capable of bringing about the destruction of the tumor cells.

MATERIALS AND METHODS

The fertile eggs used in the present investigation were from White Leghorn stock. These were incubated for intervals of 7, 9, 11, 13, 15, 17, and 19 days in a forced-draft incubator. In addition, 1-day post-hatch chicks were used. On termination of incubation the eggs were candled and divided into two groups in preparation for inoculation.

In one group a small window was made in the shell of each egg, the shell membranes were removed, and a fragment of Sarcoma 37 tissue was placed on the CAM. The hole was then sealed with Scotch tape, and the eggs were returned to the incubator. The embryos were removed from the eggs of the other group, freed from their membranes, and decapitated. Under Locke's solution buffered to pH 7.4, limbs from the younger embryos or fragments of muscle from the limbs of older embryos were removed. These fragments (or limbs), averaging about 0.2 cu. mm. in size, were placed on the culture medium containing avian egg albumen as its nutritive source. Smaller fragments of the tumor tissue were inserted into the chick limbs or muscle fragments by means of sharp needles. The culture preparations were then placed in a tissue incubator at 37.6° C. for further development. All the foregoing procedure was carried out under conditions of asepsis.

The tumor used in the course of this investigation was Sarcoma 37 obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and maintained in Rockland albino mice in this laboratory. Small fragments of relatively non-necrotic tumor tissue measuring roughly 0.5 cu. mm. were cut from transplants resident in mice for 7 days. These pieces of the sarcoma, removed from the peripheral region of the tumor under sterile buffered 0.9 per cent NaCl, were used either as grafts to the chorioallantoic membrane or as implants in chick tissue cultured in vitro.

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The preparation of the culture medium has been described by Spratt (18) with the NaCl content reduced to 143 millimolar. For organ culture of this type the albumen-agar-saline medium was poured into watch glasses where gelation occurred. The watch glasses were held in place in petri dishes by rings of cotton moistened with 0.25 per cent sodium sulfadiazine. This medium has proved to be excellent for the culture of embryonic chick tissues and adequate for mouse tumor cells of the type used in this study.

At the end of 48 hours, half of the eggs in which the CAM had been inoculated and one half of the cultured limb tissues bearing sarcoma implants were removed from incubation. The remainder of these were removed after 72 hours. Some muscle fragments from limbs of 15- and 19-day chick embryos with tumor tissue were incubated, in addition, for 5 and 7 days.

The fragments of tumors were harvested from the CAM, along with a small portion of the membrane for each. These were treated with Gerhardt's fixative and embedded for sectioning. The limb and muscle fragments containing tumor cells were treated similarly. Sections were made at 7µ and stained with DelafIELD's hematoxylin.

Table 1 indicates the number of grafts of both types used. In all, approximately 500 specimens were recovered for observation.

TABLE 1
SURVEY OF EXPERIMENTS

<table>
<thead>
<tr>
<th>Grafting site</th>
<th>Age of chick host (days)</th>
<th>No. grafts</th>
<th>No. grafts recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAM</td>
<td>7</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>Isolated limb</td>
<td>7</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>CAM</td>
<td>9</td>
<td>44</td>
<td>18</td>
</tr>
<tr>
<td>Isolated limb</td>
<td>9</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>CAM</td>
<td>11</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>Isol. limb muscle</td>
<td>11</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>CAM</td>
<td>13</td>
<td>38</td>
<td>20</td>
</tr>
<tr>
<td>Isol. limb muscle</td>
<td>13</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>CAM</td>
<td>15</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>Isol. limb muscle</td>
<td>15</td>
<td>71</td>
<td>71</td>
</tr>
<tr>
<td>CAM</td>
<td>17</td>
<td>33</td>
<td>28</td>
</tr>
<tr>
<td>Isol. limb muscle</td>
<td>17</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>Isol. limb muscle</td>
<td>19</td>
<td>53</td>
<td>53</td>
</tr>
<tr>
<td>Isol. limb muscle</td>
<td>22*</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td><strong>TOTALS:</strong></td>
<td>559</td>
<td>302</td>
<td></td>
</tr>
</tbody>
</table>

* 1-day post-hatch chicks.

Fragments from limbs of 15- and 19-day chick embryos with tumor tissue were incubated, in addition, for 5 and 7 days.

The fragments of tumors were harvested from the CAM, along with a small portion of the membrane for each. These were treated with Gerhardt's fixative and embedded for sectioning. The limb and muscle fragments containing tumor cells were treated similarly. Sections were made at 7µ and stained with DelafIELD's hematoxylin.

Table 1 indicates the number of grafts of both types used. In all, approximately 500 specimens were recovered for observation.

RESULTS

Fate of grafts to the chorioallantoic membrane.—The tumor cells in successful transplants to the CAM of embryos of all ages studied were essentially all viable when examined at the end of 2 and 3 days (Figs. 1 and 2). Within this short interval of time the cells had increased appreciably in size as compared with those removed from mice. This increase in size of tumor cells cultured in association with chick tissue has been noted before by Kauz (6) and Karnofsky and co-workers (7). The cells retain essentially a spherical shape, although some pleomorphism is evident. As in cells cultivated in the mouse, cytoplasm is scanty.

A standard feature of the tumor cells that distinguishes them from those of the chick is their deeply stained nuclei. This criterion was particularly useful in distinguishing donor from host cells in *in vitro* cultures. The size of the cells in CAM grafts also served as a helpful criterion in the determination of tissue source.

Further evidence of the viability of the tumor tissue may be seen in Figure 2, in which cancerous infiltration into the CAM substrate is evident. In addition, mitoses are far more frequent in these cells than in tumor cells cultivated in the mouse host.

There is no doubt, then, that Sarcoma 37 tissue will survive and grow on the chick chorioallantoic membrane, at least until the 20th day of incubation (17 plus 3 days). There is no appreciable evidence of necrosis in the graft, in spite of the facts that some chick macrophages are found in the CAM tissue and that vascularization of the graft has become established.

_Fate of tumor implants in explanted chick isolates._—While the picture visualized in chorioallantoic grafts is one of compatibility, this is not true for implants of tumor cells cultured *in vitro* in contact with chick muscle cells.

Implants of Sarcoma 37 in limbs or muscle fragments from younger chick embryos (7 through 15 days of incubation) survive and flourish to a limited extent for at least 3 days, the period covered in this study. Figure 3 shows the general nature of the graft in a fragment of muscle from a 15-day chick embryo cultured *in vitro* for 48 hours. The cells are more numerous in peripheral regions, indicative of compatibility with neighboring heterologous tissue. Cells in the body of the graft resemble the native tumor stock in general size and form, whereas those in invasive areas into the chick musculature are considerably enlarged. In this respect they are similar to those grown on the CAM. Moreover, mitoses are more frequently seen in the peripheral regions of the tumor, suggesting provocatively a parasitic relationship between host and donor tissues.

There is evidence of a toxic effect, however, when 17-day chick musculature serves as the host tissue. Tumor implants start to show degenerative changes as early as 48 hours after grafting. Fragmentation or clumping of the nuclear material is
the most obvious necrotic feature observed in these cells and occurs first in those nearest the avian musculature. After 3 days, degenerating cells are found throughout the graft. Similar destruction is observed in tumor implants in explanted muscle from the limbs of 19-day chick embryos, when cultured in vitro for 5 and 7 days.

Two things are of importance in this connection. Death of the tumor cells spreads from peripheral to central regions of the graft in sharp distinction to the viability of cells of implants in younger chick tissue. Moreover, there is no obvious active participation of macrophages, except perhaps in chick tissues surrounding the tumor mass. What destructive influence they may exert over short distances is not revealed by this type of study, but there is no evidence of any appreciable number of macrophages located within the implant.

Destruction of tumor cells by 1-day post-hatch chick muscle fragments in vitro is both rapid and complete. There is no invasion of the host tissue; in fact the tumor implant is effectively walled off from the muscle fibers. By 48 hours most of the cells show the toxic effect of residence in a foreign tissue, again with destruction progressing centrifugally (Fig. 4). After 3 days in culture the implant consists largely of ill-defined cells lacking nuclei.

A corollary experiment was performed to determine whether the incompatibility of the older chick tissues was a feature that developed within it autonomously or was imparted to it by other parts of the organism. To test this, implants of the sarcoma were placed in muscle fragments from the limbs of 15-day embryos and cultured in vitro for a period of 1 week. An examination of the cultures at the end of 5 days and 1 week indicated that by this time the chick tissue had become a hostile environment for the tumor cells. It appears likely, therefore, that the property of incompatibility develops in time within the fragment, even when such fragment is isolated from the remainder of the embryo. This is not the effect of an inadequate medium on the tumor cells over this extended period of time, because tumor clumps cultured alone on the albumen medium survived well and proliferated rapidly over a similar period. Neither is it a result of culture in contact with any chick tissue during this time, because the same sarcoma cells could be successfully cultivated in 7-day chick limb explants for intervals of 1 week. At termination of culture in these young chick limbs, the tumor cells were still capable of (a) producing tumors when transplanted back into mice and (b) proliferating on further passages into other chick limbs of similar age (7 days).

DISCUSSION

The present experiments have demonstrated that the musculature of chicks as young as 17 days is capable of destroying mouse tumor implants, when the muscle fragments are cultured in vitro. No such capacity has been displayed by the intact CAM of living embryos of similar age over the same brief interval of time. That this primary response of muscle elements is not of an immunological nature is indicated by the fact that there is considerable necrosis evident in tumor cells implanted in the muscle fragments for as short a period of time as 48 hours. This is further supported by the realization that there is no circulating blood in the explanted tissue.

The observation has been made that Sarcoma 37 cells can survive and proliferate in isolated limbs of 7-day chick embryos when cultured in vitro for a period of 1 week. Moreover, it has been noted that the capacity to destroy the tumor cells develops in chick muscle fragments when they are cultured for a sufficient length of time. Tumor implants in 15-day chick muscle survive in culture for 3 days but are killed when cultured in this environment for 1 week. These observations would suggest that certain metabolites of older chick muscle cells provide an environment which is toxic to Sarcoma 37 tissue. The centrifugally directed necrosis in the older tissue explants, in contrast to the selective peripheral tolerance in more incipient host tissue, lends support to the contention that diffusible substances, produced by the chick tissue, destroy the tumor cells.

It has been observed (6) that Sarcoma 37 cells inoculated into very early blastoderms (2½ days of age) undergo a period of necrosis for 3–5½ days, after which interval the remainder recover and proliferate. Studies in this laboratory have indicated a similar regressive response with Sarcoma 37 when such cells are introduced into the limbs of 7-day embryos. However, this response involves only a small portion of the cells, quite unlike the total destruction seen in implants of this tumor in the limb muscle isolates from older embryos. Observations carried out for over 1 week on grafts in 19-day muscle fragments cultured in vitro demonstrate that there is no similar recovery in this tissue.

SUMMARY

Small fragments of mouse Sarcoma 37, taken from peripheral regions of tumors of 1 week's residence in mice, were grafted to the chorioallantoic membrane (CAM) of chick embryos varying in age from 7 to 17 days. Similar portions of tumor tissue were implanted into the entire limbs (from
younger embryos) or muscle fragments from limbs (embryos of 11 days or older). These were then cultured as isolates on a medium adopted for embryonic chick tissue growth. The purpose of this procedure was to separate to a large extent the blood and tissue environments in order to evaluate the importance of each in the first visible response of chick tissue to foreign cells. The preparations were harvested after 2 and 3 days (except in a few cases) and observed for the condition of the tumor grafts.

There was no visible destructive effect in tumors transplanted to the CAM within the time range of this study. Within 48 hours, however, necrotic events were evident in the implants cultured in 17-day chick limb musculature isolates. This destruction of the sarcoma cells was more pronounced with further incubation of the explants and when the tumor was cultured in muscle fragments from older hosts. Complete necrosis was evident in tumor cells cultured in 1-day post-hatch chick limb musculature for 3 days.

The results indicate that diffusible metabolites produced by older chick musculature, but not by the tissue of the chorioallantoic membrane of embryos of similar age or by the prospective musculature of the limbs from younger embryos, provide an environment in which the tumor cells cannot survive. Moreover, this defensive measure occurs prior to the arousal of immunological activity.

ACKNOWLEDGMENTS
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REFERENCES

Fig. 1.—General features of a Sarcoma 37 graft cultivated on the chorioallantoic membrane of a 13-day chick embryo for 48 hours. Note vascularization of the graft. ×100.
Fig. 2.—Low-power photograph of a tumor fragment cultivated on the CAM of a 17-day chick embryo for 72 hours. Note vascularization of the graft and its invasion into the chick membrane. ×150.
Fig. 3.—Sarcoma 37 implant in limb muscle of a 13-day chick embryo cultured for 48 hours in vivo. The photograph shows the general nature of the graft with cell population denser near the periphery. The beginning of tumor invasiveness is also evident. ×150.
Fig. 4.—Photograph illustrating the cyto-incompatibility between Sarcoma 37 cells and muscle tissue from a 1-day post-hatch chick cultured in vivo for 48 hours. Pyknotic nuclei are visible in cells located in the center of the graft. Note also the absence of invasion of tumor cells into host tissue. ×150.
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