A Reinvestigation of Epidermal Transplantation during Chemical Carcinogenesis

David Steimmler

Departments of Pathology and Surgery, University of Utah Medical Center, Salt Lake City, Utah 84112

SUMMARY

The possibility that epidermis which has never been exposed to carcinogen can give rise to carcinomas if it is transplanted onto carcinogen-treated dermis was raised by the experiment reported by Billingham, Orr, and Woodhouse in 1951. They treated mice topically with methylcholanthrene for 12 weeks, left them untreated for 2 weeks to allow the methylcholanthrene to disappear, and then transplanted autografts of untreated epidermis obtained by trypsinization of tail skin to superficial beds prepared in the treated areas on the thorax. Tumors apparently developed from the untreated surface epidermis at 62% of the graft sites. However, in preparation of the graft beds the bases of surviving hair follicles were left in situ. Consequently, it was possible that the tumors arose from residual treated epithelium and not from the untreated epidermal grafts. To test this possibility, the original experiment was repeated in the present investigation except that F1 hybrid mice were used as the methylcholanthrene-treated hosts and inbred parent strain mice as the donors of the untreated epidermal grafts. This provided a strong (H-2) histocompatibility marker for determining the origin of the tumors by their differential transplantability to preimmunized parent strain versus F1 hybrid recipients. Of 14 carcinomas which arose at the graft sites in the primary hosts, none grew progressively in the parent strain whereas all grew in the F1 hybrids indicating that they indeed arose from F1 host cells. These results suggest that the interpretation of the original experiment as evidence of indirect epidermal carcinogenesis is incorrect.

INTRODUCTION

When a skin carcinoma is produced by the topical application of a chemical, it is generally assumed that the primary action of the carcinogen is in the epithelium which gives rise to the tumor. However, an alternate view advanced most notably in recent years by Orr (13-15) holds that the primary lesion in epidermal carcinogenesis is in the dermis rather than in the epidermis. According to this view, the carcinoma per se is an indirect effect of carcinogen-induced dermal changes or “permutation” of the stroma.

The most widely quoted evidence for Orr’s stromal permutation hypothesis is the ingenious experiment reported by Billingham et al. in 1951 (3), which is illustrated in Chart 1. A solution of MCA2 was applied to the thorax of mice for 12 weeks. The mice then were left untreated for 2 weeks to allow all traces of the MCA to disappear from the body. Next, pieces of tail skin were removed from each mouse and separated into dermal and epidermal components by trypsinization, and the pure epidermal components were autografted to the MCA-treated sites on the thorax. Papillomas or carcinomas developed at 13 of 21 or 62% of the graft sites within 265 days after grafting, and the investigators concluded that epidermis which had never been treated with MCA underwent tumorigenesis under the influence of the carcinogen-treated dermis.

Unfortunately, this simple interpretation is complicated by 2 considerations. First, since “white mice of mixed stock” were the subjects, Billingham et al. were obliged to use autografts to circumvent the allograft reaction. Hence, even though the MCA solution was applied to the thorax and the epidermal autografts were prepared from tail skin, the possibility that tail epidermis was exposed inadvertently to the carcinogen during the 12 weeks of treatment cannot be excluded. Second, and most critical, in order to graft the pieces of presumably untreated epidermis onto treated dermis, it was necessary to prepare “half-thickness” beds in which most of the dermis was left intact. Consequently, the deeper parts of surviving hair follicles remained in the beds, and it was possible that the tumors arose from residual epithelium that had been exposed to the carcinogen and not from the grafts. The investigators were fully aware of this possibility, but on histological grounds they concluded that in most cases the tumors arose from the epidermal grafts. However, uncertainty on this point has prevented general acceptance of their results as decisive evidence of indirect carcinogenesis (10).

Obviously, the critical question in the experiment of Billingham et al. is whether the tumors arose from the epidermal grafts or from residual epithelium in the graft beds. Following a suggestion of Furth (7), this reinvestigation was designed expressly to decide between these 2 alternatives. The original experiment was repeated except that F1 hybrid mice were used as the MCA-treated hosts and inbred parent strain mice as the untreated epidermal graft donors. This innovation not only excluded the possibility of inadvertent exposure of...
Epidermal Transplantation during Carcinogenesis

1. 0.3% MCA APPLIED TO THORAX OF OUTBRED MICE ONCE PER WEEK FOR 12 WEEKS

2. MICE LEFT UNTREATED FOR 2 WEEKS

3. PORTION OF TAILSKIN REMOVED; EPIDERMIS SEPARATED FROM DERMIS WITH TRYPsin AND GRAFTED TO MCA-TREATED SITE

4. 13/21 (62%) MICE DEVELOPED PAPILLOMAS OR CARCINOMAS AT GRAFT SITE WITHIN 265 DAYS

Chart 1. Experiment of Billingham, Orr, and Woodhouse, 1951.

the epidermal grafts to the carcinogen but, most importantly, provided a strong histocompatibility (H-) marker for determining the origin of tumors. If the tumors arose from the parent strain grafts, they should have grown progressively in mice of that strain as well as in the F₁ hybrids. On the other hand, if they arose from residual F₁ host epithelium, they should have grown only in the F₁ hybrids and not in parent strain mice, particularly if the latter were preimmunized to the foreign H antigens of the F₁ hybrids.

MATERIALS AND METHODS

Tumor Production. The hosts were highly inbred female mice of strains BALB/c (hereafter called C), C57BL/6 (B6), and C57BL/6 X BALB/c F₁ hybrids (B6CF₁). Except for this use of inbred mice and the change in the trypsinization procedure noted below the materials and methods of Billingham et al. were duplicated as closely as possible, and the reader is referred to their publication (3) for any technical details not given here. The 0.3% MCA in acetone was applied to the right lateral thorax with a glass pipet at a dose of approximately 0.04 ml (0.14 mg of MCA) per weekly application. At first, the fur was clipped before each application, but after several weeks of treatment little remained and clipping was discontinued. However, as noted by Billingham et al. (3), the treated areas were never epilated completely. Epidermal grafts were prepared by floating pieces of full-thickness donor tail skin on a 0.5% solution of commercial trypsin (Microbiological Associates, Inc., Bethesda, Md.) buffered to pH 7.5 with 0.1 N NaOH, at 4° for 3 hr. The skin then was rinsed thoroughly in fresh 0.9% NaCl solution, and the dermis was peeled off with watchmakers' forceps leaving behind intact sheets of surface epidermis. A single piece about 8 X 12 mm was transplanted to each recipient. The graft beds were prepared by slicing off the relatively thick and hairless surface epidermis at the treated sites with a No. 11 scalpel blade. The dressings and plaster bandages were those described by Billingham (1). They were removed 3 weeks after grafting at which time the typical tail skin cuticular "ghosts" described by Billingham et al. (3) were evident. The graft sites were inspected several times per week thereafter for the appearance and position of tumors. Chart 2 illustrates these procedures for the C to B6CF₁ donor-recipient strain combination. The resulting tumors could have had either C (H-²d) or (H-²bd) H-antigens depending on whether they arose from graft or host epithelium, respectively. In addition, untreated tail skin epidermis was transplanted to MCA-treated dermal sites in the C to C and B6CF₁ to B6CF₁ combinations. Since these were isografts, the resulting tumors could have had only C and B6CF₁ H antigens, respectively, regardless of their origin. The latter 2 groups provided tumors of known H phenotype for use as controls in the discriminative transplantation test described below.

Tumor Transplantation. Two important variables were considered in designing the test to determine tumor origin on the basis of differential transplantability to H-2-compatible and -incompatible recipients. First, it was necessary to demonstrate that the tumors were indeed capable of progressive growth on 1st passage to compatible recipients. Second, since chemically induced mouse skin carcinomas can have tumor-specific transplantation antigens which might inhibit their growth in otherwise isogeneic mice (16), it was necessary to ensure that failure of progressive growth would be due to H-2 or allograft immunity rather than to tumor-specific immunity. These variables were minimized by taking advantage of the well-known differential sensitivity of preexisting versus primary immunity to sublethal radiation (5).
Panels of normal C and B6CF₁ mice were inoculated s.c. with suspensions of about 50 million B6 spleen cells, a dose and route known to induce strong immunity to H-2-incompatible tumor allografts (2). Ten days later, when anti-B6 immunity presumably was maximal in the C recipients, the mice were exposed to 350 R whole-body radiation from a conventional 250 kV-peak X-ray therapy unit. Presumably, the sublethal irradiation would have markedly reduced if not abolished their ability to mount a primary response to tumor-specific antigens when they were challenged the next day with the tumor transplants whereas the preexisting H-2 immunity would be relatively intact. Chart 3 illustrates the discriminative transplantation test for the tumors of unknown H phenotype (C or B6CF₁?). In addition, other panels of identically prepared C and B6CF₁ mice were challenged with the tumors of known H phenotype (C, B6CF₁) to test the transplantability of these tumors to H-2-compatible and -incompatible recipients. Each tumor was transplanted to 4 C

The B6CF₁ recipients of course would not be immunized by B6 spleen cells. However, they were inoculated to control all possible nonspecific effects of the procedure such as trauma, infection, etc.
and 4 B6CF₁ recipients by depositing 2 or 3 1- to 2-sq mm pieces s.c. in the nucha with a No. 13 trocar; a piece also was saved for histological examination. The tumor implantation sites were checked weekly, and the diameters of all palpable growths were recorded. "Progressive growth" was defined as progressive increase in mean tumor diameter beyond 4 mm; in most cases, it resulted in death of the host within the maximal observation period of 14 weeks.

RESULTS

Tumor Production. The incidence of carcinomas in the 3 donor-recipient strain combinations is indicated in Table 1. The C to C combination had the lowest incidence (79%), the F₁ to F₁ had the highest (91%), and the C to F₁ was intermediate (82%). However, none of the differences in incidence or latent period are significant statistically. Four of the 18 tumors in the C to F₁ group arose on the margin of the graft sites and were discarded. However, the weekly record indicated that the 14 other tumors arose well within the grafted area, and these were chosen along the 14 tumors from each of the other 2 groups for the transplantation test. Histological analysis confirmed that all the transplanted tumors were squamous cell carcinomas.

Tumor Transplantation. The results of the transplantation tests are given in Table 2. Thirteen of 14 (93%) of the known C tumors grew progressively in 1 or more of the C test recipients and 12 of 14 (86%) in the F₁'s. In contrast, none of the known F₁ tumors grew in any of the C's, whereas 11 of 14 (79%) grew progressively in 1 or more F₁'s. The behavior of the 14 tumors of unknown H phenotype was most conclusive of all; none grew in the C's whereas all grew in at least 1 of the F₁ test recipients. A comparison of the behavior of the 3 groups of tumors strongly suggests that those in the unknown group were in fact of B6CF₁ H phenotype, i.e., that they arose from epithelium of the primary F₁ hosts and not from the parent strain epidermal grafts.

DISCUSSION

The dependence of the epidermis on the dermis for its normal morphogenesis and regional specification is well known (4, 20), and it is quite reasonable to assume that degenerative changes in the dermis have profound effects on the epidermis. The question in the present context is whether these effects might include neoplasia. Although morphological and histochemical changes in the dermis during chemical carcinogenesis have been documented extensively (11, 12, 17, 19), there is only speculation as to which if any of these changes might mediate epidermal carcinogenesis (8, 15, 19).

The only significant attempt to test the dermal mediation hypothesis critically was reported by Billingham et al. in 1951 (3). The essential feature of their investigation was the reciprocal transplantation of pieces of epidermis between carcinogen-treated and untreated areas on the same mice. Whereas no tumors at all arose at the sites of untreated dermis grafted with treated epidermis, a significant number of papillomas and carcinomas arose at the treated sites grafted with untreated epidermis. As a whole, these results suggest that carcinogen-induced dermal changes are both necessary and sufficient for epidermal tumorigenesis.

However, in spite of the elegance of these experiments, their power to support the dermal mediation hypothesis is significantly weakened by several theoretical and procedural considerations. The observation that epidermis exposed in situ to a complete carcinogen did not give rise to tumors when transplanted orthotopically to untreated dermis is after all negative evidence and as such does not exclude the possibility that under different conditions tumors might have developed from the treated grafts. For example, the epidermal grafts consisted of pure surface epidermis without pilosebaceous units, and as emphasized recently by Giovanella et al. (9) these structures may be essential for skin carcinogenesis in mice. Furthermore, even if it were possible to show conclusively that treated epidermis is incapable of tumorigenesis in association with untreated dermis, this would prove that

<table>
<thead>
<tr>
<th>Strain combination</th>
<th>No./no. mice treated (%)</th>
<th>Mean</th>
<th>Range</th>
<th>H phenotype</th>
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<tbody>
<tr>
<td>C to C</td>
<td>15/19 (79%)</td>
<td>22.3</td>
<td>19–28</td>
<td>C</td>
</tr>
<tr>
<td>B6CF₁ to B6CF₁</td>
<td>20/22 (91%)</td>
<td>22.9</td>
<td>20–28</td>
<td>B6CF₁</td>
</tr>
<tr>
<td>C to B6CF₁</td>
<td>18/22 (82%)</td>
<td>24.4</td>
<td>19–29</td>
<td>C or B6CF₁ ?</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>H phenotype of tumors</th>
<th>Number which grew progressively in:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>C recipients</td>
</tr>
<tr>
<td>C</td>
<td>1 2 4</td>
</tr>
<tr>
<td>B6CF₁</td>
<td>14 0 0</td>
</tr>
<tr>
<td>C or B6CF₁ ?</td>
<td>14 0 0</td>
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carcinogen-induced dermal changes are necessary for epidermal carcinogenesis but not that they are sufficient, i.e., that the primary lesion is in the dermis. For example, Dawe et al. (6) have shown that the presence of mesenchyme is essential for the neoplastic transformation of mouse salivary gland epithelium by polyoma virus just as it is essential for the normal morphogenesis and differentiation of salivary epithelium. However, this does not prove that the initial viral lesion resulting in epithelial tumors is in the mesenchymal component of the gland.

The other intriguing result of the Billingham et al. investigation (3), namely that epidermis which had not been exposed to carcinogen apparently did give rise to tumors when transplanted to treated dermis, is of course potentially powerful evidence for the dermal mediation hypothesis. However, as already indicated, the interpretation of this result is complicated by 2 procedural considerations; the use of epidermal autografts that inadvertently might have been exposed to carcinogen and, most critically, the possibility that the tumors arose from residual treated host epithelium and not from the untreated grafts. The results of this reinvestigation, which was designed expressly to distinguish between these last 2 alternatives, strongly suggest that the former was the case. If so, the interpretation of the original experiment as evidence of indirect carcinogenesis is incorrect. These results do not generally invalidate the dermal mediation hypothesis. However, they do demonstrate that the theory still lacks critical experimental verification.

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REFERENCES

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David Steinmuller


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