Special Property of the Cheek Pouch in Heightened Susceptibility to Heterografts of a Mouse Leukemia*

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SUMMARY

Intravenous administration of $1.0 \times 10^7$ AK-4 cells or AKR spleen cells to normal Syrian hamsters (not conditioned with cortisone) resulted in heightened immunity to cheek pouch challenge of $1.0 \times 10^7$ AK-4 cells from 2 through 6 weeks following prior exposure. However, about 50 per cent of similarly exposed animals, concomitantly treated with 2.5 mg. cortisone acetate twice per week (starting from the time of prior exposure to the cells), supported the growth of subsequent cheek pouch-challenging inocula. Heightened immunity in noncortisonized hosts manifested itself as accelerated rejection and as failure of vascularization, or implantation, or both, of the challenging inocula. Heightened susceptibility in cortisone-treated hosts manifested itself as progressive growth for 15 days, followed by death with large, necrotic cheek pouch tumors at 4-6 weeks. Growing tumors did not disseminate but retained the capacity to do so upon retransplantation into the mouse strain of origin.

The successful growth of second-set heterografts of AK-4 in cortisone-treated hamsters is demonstrated to be dependent upon a special property of the cheek pouch, by the failure of challenging subcutaneous implants to grow in suitably pretreated hosts, and by the successful growth of tumors transplanted to heterotopically autografted cheek pouches. Failure of challenging inocula to grow in suitably pretreated animals challenged in autografted full-thickness skin grafts indicates that successful growth in heterotopically grafted pouches is not a function of the surgical grafting procedures. Transferrability of the growth-supporting property of the cheek pouch with the heterotopically grafted cheek pouch indicates that the property is inherent in the cheek pouch tissues. “Immunological privilege” of the cheek pouch is discussed, and the relation of heightened susceptibility to tumor homograft enhancement is considered.

In previous work (4) the regression of cheek pouch heterografts of a mouse leukemia (AK-4) in cortisone-treated hamsters was found to result in a heightened susceptibility to challenging grafts of the neoplasm. The immunological basis of this heightened susceptibility was unclear. Regardless of the precise immunological nature of heightened susceptibility, however, it is possible to evaluate the role of the cheek pouch, since, under the previously stated conditions of dosage, the induction of susceptibility depended upon prior exposure of the host to the prospective challenging cells.

Thus, the function of the pouch may be ascertained by sensitizing cortisone-conditioned hamsters with cell suspensions of AK-4 and by challenging them via routes other than the cheek pouch. Further, it is possible to determine whether special transplantation properties are inherent in the pouch tissues by challenging suitably pretreated hamsters in heterotopically grafted pouches. The tissue specificity of the response may also be evaluated by comparing the reaction to pretreatment with leukemic AK-4 cells and normal AKR spleen cells.

The following experiments were, therefore, de-
signed (a) to induce a state of heightened susceptibility in cortisone-treated hamsters by prior exposure of the hosts to leukemic AK-4 cells, or normal AKR spleen cells, by the intravenous rather than the cheek pouch route and (b) in such animals to compare the transplantability of challenging inocula of the neoplasm in the in situ pouch, the dorsal subcutaneous space, the cheek pouch heterotopically autografted to the dorsum, and (as a surgical control for the latter), in orthotopic skin autografts.

MATERIALS AND METHODS

Intravenous pretreatment of hamsters with $1.0 \times 10^7$ AK-4 leukemic cells or AKR normal spleen cells was accomplished by retro-orbital puncture. Cortisone$^1$ therapy was initiated at the time of intravenous inoculation with cells and was maintained through challenge with leukemic cells to the conclusion of the experiments. Controls were treated with either leukemic cells or normal spleen cells but not with cortisone, since it was pertinent to determine whether pretreatment with cells alone by the intravenous route results in heightened immunity. No “cortisone-controls,” however, were included in these experiments, since previous studies (4) had shown that extensive pretreatment with cortisone alone does not result in progressive growth of challenging implants of AK-4. Experimental and control animals were challenged with $1.0 \times 10^7$ AK-4 leukemic cells or normal AKR spleen cells. Normal

planting such suspensions to in situ pouches (9).

Heterotopic transplantation of the cheek pouch is performed in these laboratories by a technic similar to that previously described for full-thickness skin homografting in the hamster (1) with minor modifications to permit manipulation of the pouch membrane.

The host was anesthetized by intraperitoneal administration of Nembutal and was denuded of its dorsal fur with blunt scissors. The denuded area was washed with a dilute tincture of iodine and was permitted to dry thoroughly. A full-thickness circular graft bed, measuring 2 cm. in diameter, was cut from the posterior dorsal skin with sharp surgical dissecting scissors. In orthotopic autografting of the skin, the circlet of removed skin was merely rotated 180° and sutured in place. In heterotopic grafting of the cheek pouch, however, the circlet of skin was discarded to create a site for the autologous cheek pouch graft.

The donor pouch was everted, thoroughly washed with aqueous Zephiran,$^3$ excised by a transverse incision across its base with sharp scissors, and placed in a sterile Petri dish. A flat membrane was easily prepared from the excised pouch by folding outward from another incision made from any point on the base toward the blind end. The presenting surface was then the connective tissue layer, from which excess connective tissue and skeletal muscle may be removed. A circular graft measuring 2 cm. in diameter was then cut from the excised pouch and sutured into the host graft bed, epithelial layer outermost, with No. 14 or No. 16 half-curved, cutting-edge needles, and 5/0 Deknatel silk. A running suture was sufficient, and no pressure bandage was required. Although surgically treated hamsters have been housed together with no ill effect, it is perhaps better practice, with cheek pouch membrane grafts, to house them separately.

Although independently devised, the technic appears to differ very little from that described by Billingham et al. (6, 7). The essential differences are that the graft site in these studies is the dorsum, rather than the chest wall, and antibiotics are omitted in washing the pouch preparatory to grafting. The gross appearance of cheek pouch grafts and the course of events following transplantation are as described by these authors.

RESULTS

In situ pouches.—Table 1 compares the effect of intravenous pretreatment with either leukemic AK-4 cells or normal AKR spleen cells. Normal

$^1$ Cortisone acetate (Upjohn), 25 mg/cc in sterile aqueous suspension. The dosage in these experiments was 2.5 mg. subcutaneously, twice a week.

$^2$ Veterinary Nembutal Sodium (Abbott), 60 mg/ml.

$^3$ Aqueous Zephiran, 1:750.
and cortisone-conditioned hamsters, pretreated with either of these cells, were challenged with inocula of $1.0 \times 10^7$ AK-4 cells in the in situ cheek pouch. The results are comparable to previous results with animals pretreated via the cheek pouch with leukemic cells (4).

As in previous experiments, control animals pretreated with cells but not cortisone not only failed to support the growth of challenging inocula of AK-4, but exhibited what has been previously distinguished (4) as a heightened refractoriness (immunity) to challenge. There was no evidence of implantation of challenging inocula in about half the instances, and in the remainder there was failure of vascularization and no increase (even temporary) in the size of the implant. Those inocula which did implant, but which did not vascularize, took 5–7 days to complete disappearance from the pouch, as opposed to the 10–14 days usually required for vascularized first-set grafts (4). Thus, of 105 hamsters pretreated with leukemic or normal spleen cells alone, all showed heightened immunity. No difference in response was detected between leukemia-pretreated, or spleen-pretreated hosts, and in both groups heightened immunity was detectable from 2 to 6 weeks following primary exposure.

Cortisone-conditioning in conjunction with intravenous pretreatment with AK-4 cells or normal AKR spleen cells resulted in heightened susceptibility in 55 per cent of 292 hamsters, as manifested by progressive growth of challenging inocula until death. Spleen cells and leukemic cells were equivalent in their capacity to elicit heightened susceptibility. Eighty-one of 144 animals pretreated with leukemic cells and 81 of 148 animals pretreated with spleen cells exhibited heightened susceptibility. The susceptibility was observable at all periods of challenge from 2 to 6 weeks following primary exposure and initiation of cortisone therapy. Tumors in animals challenged at 2 weeks had prolonged latent periods of about 14 days, in contrast to tumors in animals challenged at 3–6 weeks, in which vigorous growth was normally apparent by 5–7 days after challenge.

Growing tumors in all experimental groups behaved identically and in essentially the same manner as the tumors described in a previous report, in which the route of pretreatment was intrabuccal (4) rather than intravenous. In no instance was disseminated leukemia observed. The tumors became necrotic at about 15 days, following an inflammation-free, vigorous growth period in the cheek pouch, and by the end of the 3d week the majority had adhered so that the pouch could not be everted for observation. At death, there was evident infiltration of the adjacent structures in most animals. Biopsy of tumors prior to death revealed infiltration of the connective tissue and striated muscle of the cheek pouch. In intravenously pretreated animals, as in animals pretreated via the cheek pouch (4), death was often accomplished by marked depletion of the lymphoid tissues, hyperplasia of the bone marrow, elevated white count marked by lymphopenia and neutrophilia, and the presence of lymphocytes, neutrophils, degenerating cells, and nuclei in the lumina of the renal tubules.

**TABLE 1**

<table>
<thead>
<tr>
<th>TIME OF CHALLENGE (WEEKS)</th>
<th>LEUKEMIA-PRETREATED</th>
<th>SPLEEN-PRETREATED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortisone</td>
<td>No cortisone</td>
</tr>
<tr>
<td>2</td>
<td>15/37</td>
<td>0/9</td>
</tr>
<tr>
<td>3</td>
<td>14/17</td>
<td>0/3</td>
</tr>
<tr>
<td>4</td>
<td>17/20</td>
<td>0/15</td>
</tr>
<tr>
<td>5</td>
<td>13/24</td>
<td>0/9</td>
</tr>
<tr>
<td>6</td>
<td>24/46</td>
<td>0/19</td>
</tr>
<tr>
<td>Totals:</td>
<td>81/144</td>
<td>0/55</td>
</tr>
</tbody>
</table>

*1.0 $\times 10^7$ AK-4 cells or AKR spleen cells, intravenously, by orbital puncture.
† Interval between pretreatment and challenge with 1.0 $\times 10^7$ AK-4 cells.
‡ Results expressed as the number of animals dying with progressively growing tumor over the number of animals challenged.

Thus, the intravenous route was fully competent for immunization when cells were administered without cortisone. Similarly, the intravenous route was competent for the induction of susceptibility when the cells were administered with cortisone treatment. It may be inferred from the latter observation that the cheek pouch susceptibility noted in previous experiments (4) was not dependent upon the capacity of the pouch to block the mobility of sensitizing antigens.

**Heterotopically grafted cheek pouches.**—The results of experiments in which the route of challenge was other than the in situ pouch are given in Table 2. In these experiments tumor growth occurred in 21 of 32 hamsters challenged in their heterotopically grafted cheek pouches. No growth was observed in animals challenged subcutaneously (in a site analogous to that of the heterotopic cheek pouch graft), nor was it observed in animals challenged subcutaneously under a previously
established autograft of full-thickness skin. Again, normal spleen cells and AK-4 leukemic cells were roughly equivalent in capacity to produce heightened susceptibility.

Growth of challenging implants of AK-4 cells in heterotopically grafted cheek pouches was essentially similar to that observed in situ pouches. By 5 days small, vascular tumors were observed (Fig. 1); by 10 days growing tumors had filled the whole 2 × 2-cm. area of the grafted pouch (Fig. 2); by 20 days tumors had grown beyond the confines of the graft into the subcutaneous space around it (Fig. 3). Thereafter the tumors necrosized and in some instances sloughed, but most often animals died with large necrotic masses. Although there was evidence at death of infiltration of overlying skin, adjacent connective tissue, and underlying skeletal muscle, there was, again, no evidence of disseminated leukemia. Retention of the capacity of the cells to generalize may be inferred from the strain-specific transplantability of the tumors in AKR mice, all of which died with disseminated disease.

Thus, although it is not necessary to sensitize hamsters via the cheek pouch in order to elicit heightened susceptibility, it is necessary to challenge cheek pouch tissues in order to obtain progressive growth of the challenging inoculum. The findings indicate that a special property of the cheek pouch is required for demonstration of the susceptible state, and suggest that the pouch may function in heightened susceptibility—not by blocking the mobility of antigen but, rather, by blocking effectuation of the immune response.

**TABLE 2**

**HEIGHTENED SUSCEPTIBILITY TO AK-4 IN THE SYRIAN HAMSTER AS A FUNCTION OF TRANSPLANTATION SITE**

<table>
<thead>
<tr>
<th>Pretreating cell</th>
<th>Transplantation site</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subcutaneous intact hamster</td>
<td>Orthotopic skin autograft</td>
</tr>
<tr>
<td>AKR spleen cells</td>
<td>0/10</td>
<td>0/6</td>
</tr>
<tr>
<td>AK-4 leukemia</td>
<td>0/11</td>
<td>0/6</td>
</tr>
<tr>
<td>Totals:</td>
<td>0/21</td>
<td>0/6</td>
</tr>
</tbody>
</table>

* Transplants as inocula of 1.0 × 10⁷ AK-4 cells in saline suspension.
† One sensitizing inoculum of 1.0 × 10⁷ cells intravenously; all animals concomitantly conditioned with cortisone.

DISCUSSION

Previous experiments have established that the regression of cheek pouch transplants of a mouse leukemia, AK-4, occasions a heightened immunity toward challenging inocula of the neoplasm. The immunity was demonstrated either as a failure of intravenous challenging inocula to implant and grow following total-body x-radiation (2) or as the accelerated rejection of challenging inocula in the same or contralateral cheek pouch (4). It may be inferred that the characteristic regression of first-set grafts of AK-4 is due to the acquisition of immunity on the part of the host toward antigens of the implanted neoplastic cells. Thus, despite good evidence that the cheek pouch is an “immunologically privileged” transplantation site for homologous and heterologous skin grafts—attributed to a capacity of the pouch connective tissues to block immunological sensitization of the host (6-8, 10)—it is clear that such privilege is not normally extended to AK-4 cells.

Nevertheless, the same experiments (4) showed that a heightened susceptibility toward challenging inocula of AK-4 cells was occasioned by the regression of first-set grafts of the neoplasm in the presence of cortisone. The logical possibility was thus raised that cortisone treatment of the host may have physiologically altered the pouch in such a manner that it did indeed behave, under those circumstances, as a barrier to the immune response. It was also suggested, in the discussion of those results (4), that the described conditions may have resulted in an augmented depression of the immune response or in some variant of enhancement, despite the well known inhibitory effect of cortisone on tumor homograft enhancement in mice (11). Although the present experiments are intended principally to evaluate the role of the cheek pouch in the induction of heightened susceptibility to AK-4 heterotransplants, they tend to clarify these other hypotheses.

The successful induction of susceptibility through intravenous sensitization adequately rules out the possibility that susceptibility is attributable merely to a capacity of the pouch of the cortisone-conditioned hamster to interfere with immunological sensitization of the host. In fact, the intravenous route is somewhat superior to the cheek pouch route for the induction of heightened susceptibility to AK-4. This is evident not so much in the percentage of animals rendered susceptible but rather in the earlier appearance, at 2 weeks, of the susceptible state. The results at the present time, therefore, suggest that susceptibility is more a function of release from, rather than restriction
to, the pouch of antigenic material and the consequent stimulation of antibody-forming mechanisms.

Concerning a potentiated depression of the immune response, it is probable that antigenic stimulation in conjunction with cortisone-conditioning induced no complete collapse of the immune response. In that event, subcutaneous challenging inocula should have been expected to grow. Although the failure of subcutaneous inocula to grow implies the existence of an immune response, it nevertheless is indeterminate from these experiments whether subcutaneous immunity was the result of pre-existing levels of antibody, or the activity of an incompletely anergic antibody-forming machinery, or both.

Whatever the precise nature of the immune response which presumably inhibits the growth of subcutaneous challenging inocula in the cheek pouch-susceptible hamster, it is probable, although not as yet decisively demonstrated, that some form of the immune response is required for such cheek pouch susceptibility. In support of this hypothesis, previous experiments (4) have shown the necessity to pretreat the hosts with the prospective challenging cells, or—as in these experiments—with genetically and antigenically equivalent spleen cells (5) in order to achieve heightened susceptibility. Other experiments have demonstrated the passive transferrability of heightened susceptibility with the sera or plasma of sensitized normal, or sensitized and cortisone-conditioned hosts (5). These experiments suggest not only that humoral antibody is present during the susceptible state but that its presence may be required to elicit heightened susceptibility.

The acceptance of cheek pouch-challenging inocula by immunized animals, however, implies, ipso facto, that the pouch has the capacity to block effectuation of the immune response. From the above data, which imply the necessity for an immune response to become established in order to achieve this acceptance, it perhaps may be further inferred that the capacity of the pouch to thus act as a barrier to effectuation of the immune response is, at best, partial. The results therefore suggest that the pouch may qualitatively or quantitatively screen humoral antibody in such a manner that a noncytotoxic but antigen-inactivating reaction may take place at the graft site.

The possibility that the pouch to some extent may normally behave in this manner has been suggested elsewhere (4) in a discussion of the characteristically successful heterotransplantability of certain disseminating murine leukemias, other than AK-4. It is not clear, on the basis of still incomplete evidence, whether cortisone-conditioning renders the pouch tissue a more efficient barrier to effectuation of the immune response. It is equally possible that a lowered immune response in the presence of a chemical suppressor of immunity (cortisone) may make a partial capacity of the pouch to block the immune response relatively more effective.

In view of the “barrier hypothesis” of Billingham et al. (6, 7), it should be pointed out that the results can be explained in terms of the combined action of an immune response, profoundly depressed by cortisone and antigen (12), together with the capacity of the pouch to block antigenic stimulation by the challenging cells of the antibody-forming mechanisms. This argument, however, is tenuous, since previous experiments have established that extensive pretreatment with cortisone alone does not appear to affect the acquisition of immunity to challenging AK-4 grafts.

Further, there is no conclusive evidence, as yet, that AK-4 antigens in combination with cortisone will induce any greater depression of the hamster’s immune response than will cortisone alone.

In any event, previous experiments (4) have indicated that heightened susceptibility toward AK-4 cheek pouch heterografts is systemically inducible; it can be elicited in one pouch by pretreatment via the contralateral pouch. The present experiments confirm this finding, since sensitization via the intravenous route is fully competent to induce susceptibility. Nevertheless, it is evident that challenge via the pouch is also required to demonstrate susceptibility. This is evident not only from the failure of subcutaneous challenging inocula to grow, but from the successful growth of challenging inocula in heterotopically grafted pouches. These latter experiments further indicate that, whatever the special property of the pouch required for demonstrating heightened susceptibility to AK-4 heterografts, the special property is probably inherent in, or closely associated with, the pouch tissues.

Thus, induced heightened susceptibility to AK-4 in hamsters appears to be an immunological phenomenon dependent upon some qualification of the immune response induced by antigen in the presence of cortisone; it also appears to be a systemically inducible state with local manifestations dependent on some special property inherent in, or closely associated with, the pouch tissues. The presently available information suggests that this special property is a partial capacity of the pouch to block the access of antibody to the challenging
AK-4 cells. The nature of the mechanism by which the pouch can exert such a blockade is, as yet, unknown.

REFERENCES

Fig. 1.—Small vascular tumor in the heterotopic cheek pouch autograft of a Syrian hamster intravenously exposed to AK-4 cells 4 weeks prior to challenge and treated with cortisone up to and through the period of challenge. At 5 days following challenge, the growing tumor has half-filled the graft.

Fig. 2.—Same animal as in Figure 1, at 10 days. The challenging tumor has grown to fill the entire area of the circular cheek pouch graft and has begun to grow beyond the confines of the graft.

Fig. 3.—Same animal as in Figures 1 and 2, at 20 days. The growing tumor has grown well beyond the confines of the cheek pouch graft.
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