Heightened Immunity and Susceptibility toward Cheek Pouch Heterografts of a Mouse Leukemia in Syrian Hamsters*  

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SUMMARY

Heterotransplantation of $1.0 \times 10^7$ AK-4 cells of AKR mice into cheek pouches of normal and cortisone-treated adult Syrian hamsters resulted in measurable but transient growth; small vascular tumors which appeared at 5–7 days regressed completely by 10–14 days. Noncortisonized hamsters vigorously rejected second-set grafts, which failed to vascularize, in considerably less time, in either the previously exposed or contralateral pouch. Hamsters treated with cortisone during and following rejection of the first-set graft also vigorously rejected second-set grafts at 2 weeks following first-set grafting but accepted second-set grafts, in 50 per cent of the instances at 4–6 weeks, in either the previously exposed or contralateral pouch. This acceptance of second-set grafts was in contrast to the failure of hamsters pretreated with cortisone alone to support the growth of first-set grafts.

Successful second-set grafts in cortisone-treated hosts grew progressively for 10–14 days, after which interval the grafts necrotized and ulcerated. Despite evidence of locally infiltrating cheek pouch tumors at death at 4–6 weeks, in no instance was disseminated leukemia observed. Retransplantation of hamster-borne tumors to mice, however, resulted in selective death of AKR mice with disseminated disease, indicating retention of strain-specificity and the capacity of the cells to generalize.

The possible relation between heightened susceptibility to AK-4 heterografts in the hamster pouch, and tumor homograft enhancement in the mouse is discussed, as is the significance of this study with respect to the concept of “immunological privilege” of the cheek pouch.

Previous experiments have indicated that the “immunological privilege” of the Syrian hamster cheek pouch (as demonstrated by Billingham et al. [10–12] with grafts of homologous skin) is probably not universal (4). For example, implants of a mouse leukemia, AK-4, which characteristically fail to grow in the pouch—unlike certain other murine leukemias (20, 21)—apparently evoke an immune response. The heightened immunity occasioned by regression of such cheek pouch implants is demonstrable by the subsequent failure of quantitated intravenous inocula to implant and grow following total-body x-radiation at doses as high as 1500 r (4), whereas earlier work (5) had shown that first-set grafts of AK-4 would grow in unsensitized hamsters under those conditions of x-ray dosage and route of inoculation. Thus, despite its “immunological privilege” with respect to heterotransplants of certain murine leukemias as well as homotransplants of skin fragments, the pouch does not unqualifiedly support the growth of all transplanted tissues. The results with x-radiated hamsters suggest that those tissues which fail to grow in the pouch succumb to an immune response.

Essential to such a conclusion, however, and lacking in the above-cited demonstration that the pouch is not a “barrier” (12) to AK-4 antigens, is some indication that the immunity incited by regression of first-set cheek pouch transplants can operate against challenging inocula in the pouch.
itself. The present experiments are intended, in part, to correct that deficiency. Further, since cortisone is widely employed in promoting heterotransplantation in the cheek pouch, its effect on the host's acquired immunity has also been examined. The purpose of this study, therefore, is to investigate the rejection time and vascularity of first-set and second-set grafts of AK-4 in the cheek pouches of normal and cortisone-conditioned hamsters.

MATERIALS AND METHODS

Three hundred and seventeen hamsters were given inoculations of a first-set graft of $1.0 \times 10^7$ AK-4 cells in the right cheek pouch. Of these, 207 were given subcutaneously, in addition to the cells, twice-weekly injections of 2.5 mg. cortisone. Cortisone therapy was initiated at the time of inoculation with cells and was maintained through the period of rechallenge with a second-set graft of the cells to the conclusion of the experiment. An additional 76 "cortisone controls" received cortisone alone, prior to their first-set grafts.

At intervals of either 2, 3, 4, 5, or 6 weeks following pretreatment, hamsters of all three groups were challenged once with $1.0 \times 10^7$ AK-4 cells. Part of each group was challenged in the right cheek pouch (the pouch used for first-set transplantation), and the rest of the animals were challenged in the left (contralateral) cheek pouch. "Cortisone controls" not previously exposed to cells were challenged only in the right cheek pouch.

Young adult Syrian hamsters (8–12 weeks of age) were obtained for these experiments from a closely bred colony in which homografts of full-thickness skin enjoy long-term persistence, evoking only a low-grade, chronic, homograft reaction (1–8, 8).

AK-4 cells for inoculation as first-set or second-set grafts were obtained from the spleens and lymph nodes of carrier lines of AKR/Jax mice, as in experiments reported previously on the transplantability of this neoplasm in x-irradiated recipients. The tissues were forced through a stainless steel mesh (1/32-inch opening) into physiologic saline. Cell concentrations were then determined by standard hemocytometric methods, and suspensions were adjusted to a final concentration of $1.0 \times 10^8$ cells/ml. Inocula of 0.1 ml., containing $1.0 \times 10^7$ nucleated cells, were injected into cheek pouches with 1.0-ml. tuberculin syringes, equipped with 24-gauge needles. Inoculation of the cheek pouch with a cell suspension is accomplished under light Nembutal anesthesia, according to methods previously described (17). Trypan blue staining of several random samples of the adjusted suspensions revealed about 10-12 per cent stained cells.

Vascularization of first-set and second-set grafts was observed directly at 2- to 3-day intervals, with the host under light Nembutal anesthesia. Vascular tumors are cherry-red in color, nonvascular implants are pale. Individual growths were measured in three dimensions to the nearest millimeter, and averages of the product of these dimensions were plotted against time for each experimental group. At death, representative animals were autopsied, and the tissues were fixed in 10 per cent formalin and stained with hematoxylin and eosin for microscopic evaluation. Occasional biopsies were made prior to death for similar purposes and to test the viability of the pouch contents by retransplantation into mice.

RESULTS

First-set grafts.—The transient growth of first-set grafts of $1.0 \times 10^7$ AK-4 cells in the cheek pouches of untreated or cortisone-conditioned hamsters is illustrated in the left-hand group of curves of Chart 1. This transient growth is indicative of the hamster's normal nonsusceptibility to implants of this neoplasm. Cheek pouch inocula formed a bleb, which disappeared at 24-48 hours. In normal, as well as cortisone-conditioned hamsters, vascular implants appeared at 3-5 days, following which the grafts grew to a maximum volume at 5–7 days. Thereafter the grafts de-

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1 Cortisone acetate (Upjohn), 22 mg/cc, in sterile aqueous suspension.
2 Tumblebrook Farm, Brant Lake, New York.
creased in size, lost their cherry-red color, and completely disappeared in 12-14 days. Although tumors varied greatly in size at the peak of growth, growths in the pouches of cortisol-conditioned hamsters were, in general, smaller than those in the pouches of normal hamsters. Cortisone-conditioning initiated at the time of inoculation with cells generally did not prolong survival of first-set tumor implants.

Extensive pretreatment of hamsters with cortisol alone similarly did not significantly alter the “normal” pattern of transient growth followed by regression at 12-14 days (Table 1, Chart 1).

**TABLE 1**

HETEROTRANSPLANTABILITY OF AK-4 GRAFTS IN THE CHEEK POUCHES OF SYRIAN HAMSTERS*

<table>
<thead>
<tr>
<th>Time of Challenge (WEEKS)</th>
<th>Second-set grafts§</th>
<th>First-set grafts§ in Cortisone-Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortisone RCP</td>
<td>Cortisone LCP</td>
</tr>
<tr>
<td>2</td>
<td>0/24</td>
<td>0/24</td>
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<tr>
<td>3</td>
<td>1/15</td>
<td>1/15</td>
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<tr>
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<td>9/20</td>
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<tr>
<td>5</td>
<td>14/34</td>
<td>8/18</td>
</tr>
<tr>
<td>6</td>
<td>16/37</td>
<td>18/28</td>
</tr>
<tr>
<td>Total:</td>
<td>35/107</td>
<td>36/100</td>
</tr>
</tbody>
</table>

* All grafts as cheek pouch inocula of 1.0 × 10^7 AK-4 cells in 0.1 ml suspension.
† After transplantation of first-set graft, or the initiation of cortisol pretreatment in cortisone controls.
‡ Results are expressed as the number of growing tumors over the number of animals challenged. RCP = right cheek pouch; LCP = left cheek pouch.
§ Following pretreatment with cortisol alone for the indicated number of weeks. Normal pattern of transient growth followed by regression. All other negatives exhibited accelerated rejection. See text.

The dosage of cortisol used in these experiments is known to be adequate for the heterotransplantation of certain mouse leukemias (20, 21) or cell cultures of normal and neoplastic origin (17). Nevertheless, cortisol-conditioning does not completely suppress the immune response and, as the present experiments with AK-4 illustrate, will not permit the hamster to accept all tissue grafts. Although critical dose-response studies with cortisol have not been conducted, it is the general experience of this laboratory that higher doses of cortisol do not materially affect the nontransplantability of AK-4. Similarly, lower doses have been without effect.

**Second-set grafts in normal hamsters.**—Hamsters which had previously rejected first-set cheek pouch grafts vigorously rejected second-set cheek pouch grafts. The challenging inocula either showed no evidence whatsoever of implantation or formed small, barely measurable, pale implants which did not vascularize and which were completely resorbed by 7-10 days. This pattern of accelerated rejection was observed in either the pouch used for first-set transplantation or the contralateral pouch, from 2 weeks through 6 weeks following primary exposure. No vascular, measurable growth was found in 65 right cheek pouches, or in 47 (contralateral) left cheek pouches, or a total of 110 cheek pouches so studied.

**Second-set grafts in cortisol-treated hamsters.**—Previous rejection of first-set grafts by hamsters concomitantly treated with cortisol resulted in heightened immunity in some, and heightened susceptibility in others, to second-set grafts in the same or contralateral pouch (Table 1). Challenge at 2 weeks resulted in accelerated rejection of all second-set grafts. Challenge at 4, 5, or 6 weeks, however, resulted in progressive growth of cheek pouch tumors in about 50 per cent of the animals. During the tumor-susceptible period, non-susceptible animals manifested the same kind of accelerated rejection observed at 2 weeks, or in rechallenged normal animals at all times.

In the 50 per cent of animals which exhibited tumor growth, progressively growing tumors, free of inflammation, grew beyond the 5- to 7-day peak period observed in first-set grafts and underwent necrosis and ulceration at 10-15 days. Ultimately the tumors adhered, and the pouches could not be everted for observation. Animals dying at 4-6 weeks had large necrotic cheek pouch tumors with viable cortices and with some indication of leukemic infiltration of adjacent tissues. In no instance was disseminated leukemia observed. Biopsy of earlier tumors revealed infiltration of the loose and dense connective tissue layers and striated muscle of the pouch and, in some instances, of the pouch epithelium (Fig. 1). The tumors appeared to be free of polymorphonuclear infiltration and, similarly, necrosis was often observed to be acellular. Animals died with hyperplastic bone marrow, marked depletion of the lymphoid tissues, and renal tubules packed with degenerating cells of uncertain origin (Fig. 2). In some instances degenerating cells and nuclei were found in the liver as well. Examination of the peripheral blood in a few randomly chosen subjects revealed no leukemic cells, elevated total white counts characterized by absolute lymphopenia, and marked neutrophilia. In some instances there were large numbers of atypical histiocytes and monocytes: bi- and trilobation, folding and overlapping of the nucleus.
such immune response. Further, heterotopic homologous cheek pouch transplants of homologous skin are "privileged" in that they do not provoke any grafts of the pouch itself are similarly "privileged."

免疫反应可以观察到在囊 notifying the host to subsequent challenge with the tumor. Cheek pouch challenge of a refractory hamster has the advantage of permitting a careful examination and accurate appraisal of the accelerated rejection and associated failure of vascularization (9) characteristic of the second-set rejection of AK-4 grafts. This heightened immunity is largely evidenced by heightened refractoriness of the host to subsequent challenge with the tumor. Cheek pouch challenge of a refractory hamster has the advantage of permitting a careful examination and accurate appraisal of the accelerated rejection and associated failure of vascularization (9) characteristic of the second-set rejection of AK-4 grafts. This heightened immunity is largely systemic in nature, since regression of first-set grafts in right cheek pouches had invariably rapid and fatal consequences for second-set grafts in left cheek pouches. The results are thus somewhat reminiscent of similar observations by Greene (19) with neoplastic heterografts in another "privileged" site, the anterior chamber.

Concerning the specificity of this immune reaction other studies (4–6) indicate that the hamster's immune response to mouse leukemic grafts probably is species-specific rather than individual-specific. The absence of individual specificity in the heterograft reaction is generally accepted (cf. Brent [15], however, for a discussion of exceptions). This is perhaps to be expected from the greater taxonomic diversity that exists between species than that which exists between members of the same species.

Thus, the present studies confirm the findings of earlier work (4)—that AK-4 heterografts in the cheek pouch can incite immunity toward second-set grafts. These studies further indicate that the immune reaction can be observed in the pouch itself.

Billingham et al. (10–12), however, have shown that cheek pouch transplants of homologous skin are "privileged" in that they do not provoke any such immune response. Further, heterotopic homografts of the pouch itself are similarly "privileged."

This "privilege" may in both instances be interpreted by exposure of the host to the tissue isoantigens by routes other than the cheek pouch at any time before or after transplantation. These authors, in their "barrier hypothesis" (12), have proposed that the pouch may interfere "with the afferent pathway of the immunological reflex" (10), and in their experiments with skin the pouch appears to constitute no efferent barrier to the immune response.

It is evident, however, that the pouch is no afferent barrier to AK-4 antigens. The apparent conflict in data may be resolved by supposing that the pouch does not behave as an afferent barrier to invasive neoplastic cells, in much the same way as it is presumably no efferent barrier to sensitized lymphocytes in effectuation of the homograft response. In support of this hypothesis, Handler (20) and Handler et al. (21) already have observed the dissemination of leukemic cells into the blood and viscera from successful cheek pouch transplants of certain mouse and rat leukemias which, unlike AK-4,characteristically grow in the hamster cheek pouch. A close consideration of this fact, and the immunity incited by regression of AK-4 in the cheek pouch, indicate that the behavior of heterotransplanted murine leukemia, and possibly cell cultures of malignant origin as well (17), may not be explainable solely in terms of the pouch's acting as a barrier to immunologic sensitization of the host. Although it has been speculated (10, 16, 18) that the same mechanisms underlying homotransplantation of skin in the cheek pouch may explain the heterotransplantation of malignant cells or tissue as well, the present studies tend to indicate that such a view may not be entirely correct.

Cortisone-conditioned hamsters.—Although the results of second-set grafting in cortisone-conditioned hamsters seem to indicate that cortisone has reversed the immune response to AK-4, perhaps this is not wholly true. Cortisone treatment, concomitant with first-set grafting of AK-4, did not appear to influence the neoplasm's ultimate regression, nor did extensive pretreatment with cortisone alone alter the immune response to first-set grafts (Table 1). Other experiments (in progress) also indicate that, if immunity is stimulated in unconditioned hamsters by first-set grafting of the cells, the second-set immune response is not altered by cortisone administered along with the second-set graft. Such results are in accord with certain evidence in mice that, although cortisone, like x-ray (vide supra), may sometimes suppress the acquisition of immunity, it will not abrogate...
a transplantation immunity that has already been established.

The heightened susceptibility toward second-set grafts observed in these experiments should, therefore, be considered to be related to prior exposure of the hamster to graft antigen together with cortisone. This view is sustained by the observations that pretreatment with cortisone alone was without effect on the normal acquisition of immunity and that pretreatment with cells alone stimulated heightened immunity. The design of these experiments permits no conclusion, however, whether the observed alteration in immune status was permanent or cortisone-dependent, since experiments in which cortisone is withheld following regression of the first-set grafts have not yet been done. Nevertheless, the data suggest that (a) concomitant exposure to antigen may augment the action of cortisone in depressing the immune response or (b) cortisone may quantitatively or qualitatively alter the immune reaction in such manner that the end-result is similar to enhancement.

The results with cortisone-conditioned hamsters may bear some relation to recent reports in which partial or complete abrogation of the homograft reaction is potentiated by pretreatment of the host with antigen in combination with a chemical agent. Rubin (39) reported reversal of the immune response to 6C3HED in previously immunized DBA/2 mice by later application of methylcholanthrene; his later work has also indicated that parabiotic tolerance between widely genetically different strains can also be achieved by concomitant treatment with the carcinogen (31).

More significant for the present study, perhaps, is his observation that, although the carcinogen used alone influenced the immune response slightly, if at all, and pretreatment with donor spleen cells was ineffective, carcinogen and spleen cell suspension used together produced a greatly potentiated acceptance of challenging skin homografts. Medawar has shown (26) with skin homografts that the immune-suppressive capacity of intravenously administered semi-soluble antigen is potentiated by concomitant treatment of the host with amethopterin. Medawar is inclined to interpret these results in terms of the synergistic effect of intravenously administered soluble antigen and a chemical suppressor of immunity; but Rubin, having demonstrated passive transfer of susceptibility induced with methylcholanthrene, suggests the possibility that some variant of the enhancement response has been induced in that system.

The induced susceptibility to heterografts of AK-4 described in this report has also been shown to be passively transferable (5). Other evidence, provided by the present study, that the state may be a variant of enhancement, is suggestive, although tenuous. The evidence resides principally in certain general similarities to mouse systems: (a) the transience of AK-4 growth in hamsters under normal conditions; (b) the necessity to pretreat the host with the prospective challenging cells in order to achieve susceptibility; (c) the resultant close association with immunity; and (d) the transition from a state of heightened immortality to one of heightened susceptibility.

Militating against the assumption of enhancement is the paucity of reports in which leukemias are homologously enhanced in the mouse (32). Also, heterografted leukemias apparently have never been enhanced, although enhancement of solid mouse tumor in the rat has been achieved (28, 29). Further, enhancement (34, 35) is considered to be in response to pretreatment of the host with products of, extracts of, or antisera to the tumor, although Kaliss (23) presents examples of enhancement of tumor grafts through prior exposure of the host to live tumor transplants.

General considerations.—In alternative attempts to explain the undoubted immunological privilege of the pouch in heterotransplantation of highly antigenic (13, 14, 22, 25) malignant cells, the possibility should be considered that the pouch may interfere partially in the mobility of humoral antibody. That such interference would be, at best, partial is indicated by the results of the present experiments with normal, unconditioned hamsters, in which the pouch is obviously not a complete barrier to effectuation of the immune response. Thus, differences in the transplantability of the murine leukemias in hamsters could be regarded as due to differences in the invasiveness, or antigenicity, or both, of the transplanted cells. It is also possible that disseminated leukemic cells may overwhelm the antibody-forming centers before an immune response can become established.

The results of the present experiments with AK-4-sensitized, cortisone-treated hamsters, however, may suggest that aside from or in addition to the immune suppressive properties of cortisone, conditions are set up under which some self-enhancement of the graft may also take place. It is also possible that cortisone treatment of the host may physiologically alter the pouch tissues so as to make them indeed a barrier to the immune response. A further study will investigate the participation of the cheek pouch in the induction of heightened susceptibility to AK-4.

The need for more information on mechanisms
whereby a host will support an antigenically variant tumor, of which enhancement may be considered one example, is made more pressing by recent experiments of Prehn (30) and Sjögren et al. (38). These experiments demonstrate the antigenicity of chemically and virally induced tumors in mice and pose the problem of how such tumors survive in the autochthonous host without evoking an auto-immune "homograft" response. A theoretical solution to the problem may be available in the concept of self-enhancement, which, at least, must be ruled out in any explanation of the seemingly paradoxical phenomenon. Further elucidation of the mechanisms underlying heterotransplantation in the hamster pouch, and heightened susceptibility, may contribute useful information.

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**Fig. 1.**—Growing second-set graft of AK-4 cells in cortisone-treated hamster. Biopsy of 12-day cheek pouch tumor. Infiltration of connective tissue and epithelium of the pouch. Hematoxylin and eosin, ×275.

**Fig. 2.**—Kidney of cortisone-treated hamster bearing a second-set AK-4 cheek pouch tumor; 29 days. Note tubules containing degenerating cells and nuclei. Hematoxylin and eosin, ×115.

**Figs. 3-5.**—Inflammatory cells in the peripheral blood of cortisone-treated hamsters bearing second-set AK-4 tumors in cheek pouch. Wright's stain and Giemsa, ×1010.
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