THE ROLE OF THE CHEEK POUCH IN EFFECTING TRANSPLANTATION IMMUNITY IN THE HAMSTER*

BY DAVID SHEPRO, PH.D., NORA KULA, AND JAMES A. E. HALKETT
(From Simmons College and Boston University Biological Science Center; Radioisotope Service, Boston Veterans Administration Hospital, Boston)

(Received for publication January 7, 1963)

“Atypical,” “privileged,” “unique” are but a few of the many terms utilized by investigators in their descriptions of the hamster cheek pouch as a site for the transplantation of normal and abnormal tissues. The commentaries are based upon the known phenomenon that an unusually large variety of homografts and to a lesser extent heterografts will survive in the cheek pouch. In addition, homografts which are rejected when transplanted orthotopically frequently will “take” when placed within the cheek pouch. Finally, as observed in our laboratory, certain tumor transplants grow more rapidly within the cheek pouch than the same tumor transplanted in other areas; e.g., flank or ear.

Billingham et al. (1960) presented data which indicated that the privileged status of the pouch was derived from “—a layer of loosely packed areolar tissue,” the major layer of the membrane. Likewise, our opinion has been that the uniqueness of the cheek pouch was due primarily to some structural aspect of the membrane. In addition, we were inclined to believe that the “inhibiting effect” of the cheek pouch was directly related to its alymphatic nature; hence in order to reach the regional draining node, tissue antigens would have to traverse the connective tissue meshwork of the membrane before reaching the site of the immunological mechanism. Therefore, a series of experiments was devised to test this “barrier” hypothesis, namely that the areolar connective tissue prevented or hindered the passage of large molecules. A secondary feature of these experiments is that the results would also provide further information regarding the relationship between the lymphatic system and the cheek pouch. Recently Miotti (1961) published an excellent monograph on the lymphatic system of the hamster. However, no mention was made of the lymphatic system in the cheek pouch except for an explanation beneath a diagram that states that the “submental node drains the cheek pouch.” This omission or oversight regarding the lymphatic drainage of the cheek pouch

* This investigation was supported by United States Public Health Service grant # AI-03767-03.
was especially noticeable because Miotti treats the lymphatic drainage of all other areas of the hamster body with great skill and in great detail.

**Materials and Methods**

In all experiments, golden hamsters (*Mesocricetus auratus*) of both sexes, bred at random weighing between 40 and 70 gm, were employed. The animals were housed 2 to a cage and maintained in a routine animal room manner. Materials used for the trace studies were India ink, colloidal chromic radiophosphate, and thorotrast; details of each procedure are presented below. The animals were anesthetized with nembutal and the everted cheek pouches (the right side only) were injected through a 30 gauge needle attached to an adapter polyethylene tube syringe. This unit provides more maneuverability for injecting the delicate membrane than a needle attached directly to the syringe. With a stereoscope, we were able to inject from 0.05 to 0.5 ml accurately without apparent leakage or injury to the blood vessels. A good injection resulted in a large bubble forming in the membrane plus a temporary constriction of the adjacent vessels. These signs indicated that the injected materials were flowing within the connective tissue layer of one wall and not between the connective tissue layers of opposite walls of the everted pouch. A large blood vessel was used as a marker to approximate the same injection area for all animals. Injections into the pinnas and into the lips of the control animals were performed in a similar manner. The right side again was arbitrarily selected as the injection side.

*India Ink.*—India ink (Carters) diluted with distilled water 1:4, was injected into the everted cheek pouches of 6 animals to observe the movement of dye through the membrane. The quantity of dye varied between 0.2 and 0.5 ml. The cheek pouches were examined at intervals for a period of 6 to 8 hours. The regional cervical nodes were also exposed to check for the appearance of dye within the lymphatic tissue or within the afferent lymphatic vessels.

A second series of dye injections was made, this time following a priming of the membrane connective tissue with hyaluronidase (Nutritional Biochemical Corporation, Cleveland). The hyaluronidase (0.1 ml containing 300 Ω.S.P. units) was injected 3 hours before the dye.

*Chromic Radiophosphate.*—Four hamsters received 0.05 ml of P³² (Abbott Laboratories, North Chicago, assay = 1.40 mc/ml) injected into the everted cheek pouches. An equal volume of radioactive phosphate was injected into different sites (lips, pinnas, hearts) of 9 control animals. All animals were killed 24 hours postinjection and the regional superficial cervical nodes draining these sites, along with the contralateral nodes, spleen, thymi, and samples of liver, were removed. The tissues were weighed and prepared for liquid scintillation utilizing hyamine (Packard Instruments Co., Inc., La Grange, Illinois) to dissolve the tissues and toluene and liquifluor (Pilot Chemical Co., Inc., Long Island City) as the scintillating medium. The procedure was essentially the same as outlined by Herberg (1960). All samples were counted in a Nuclear-Chicago 3 channel liquid scintillator counter for 5 minutes. Background counts were subtracted from the experimental counts and when necessary values were corrected for decay. Tissue samples are expressed as counts per minute per milligram (c.p.m./mg).

An extended study of the fate of P³² injected into the cheek pouch was performed on 12 additional animals. The procedure was the same as outlined in the above paragraph except that 4 animals were killed at weekly intervals for 3 weeks.

*Thorotrast.*—0.05 ml of thorotrast (an aqueous suspension of thorium dioxide and dextrin) was injected into the cheek pouches of 6 hamsters. The animals were x-rayed at intervals ranging between 1 hour and 7 weeks. The x-rays were made at 20 ma, with a Keleket x-ray machine, 42 kv, for 0.5 second with an extension cylinder cone placed 24 inches above the animal. Ultrafast Dupont medical x-ray film (Anasco, Binghamton, New York) was used.
RESULTS

India Ink.—Injections of India ink alone were unsuccessful in demonstrating lymphatic vessels or producing any change that would illuminate the problem of how particulate matter escapes from the pouch. However, ink injections coupled with hyaluronidase resulted in a rapid spread on an "ink front" within the connective tissue of the pouch membrane. Again, no discrete vessels could be seen but the pathway of the ink leaving the pouch was established. The particulate matter travels toward the mouth down the connective tissue of the neck and stops rather abruptly about 1 cm above the regional superficial cervical node. Small blackened afferent lymph vessels were seen entering the node from the stained area. The length of time needed for the ink to reach the node varied with the amount injected, but blackened nodes were always visible within 2 to 3 hours.

Chromic Radiophosphate.—At the end of the 1st day, the regional nodes draining the lips (superficial cervical node) and the pinnas (auricular node) showed high radioactivity. The mean counts of these nodes were 83,350 cpm/mg tissue and 118,000 cpm/mg tissue respectively (Table I); the contralateral nodes produced mean counts of only 223 cpm/mg tissue and 263 cpm/mg tissue. The regional nodes draining the experimental cheek pouch injections, in contrast, produced a mean count of only 138 cpm/mg tissue. Excluding the lip and ear regional nodes plus the spleen and liver of the cardiac controls, each group of tissues reported in Table I had similar low levels of radioactivity and all of the counts fell within a rather narrow range. Following removal of the organs, the animals were scanned with a Geiger-Müller tube. No appreciable activity above background was recorded in any parts of the control animals. The same was true for the experimental hamsters with one notable, important exception, the activity in the cheek pouch. When a fragment of the

<table>
<thead>
<tr>
<th>Site of injection</th>
<th>No. of Animals</th>
<th>Superficial cervical node</th>
<th>Right auricular node</th>
<th>Thymus</th>
<th>Spleen</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheek pouch</td>
<td>4</td>
<td>138</td>
<td>169</td>
<td>34</td>
<td>145</td>
<td>189</td>
</tr>
<tr>
<td>Lip</td>
<td>3</td>
<td>83,350</td>
<td>223</td>
<td>104</td>
<td>263</td>
<td>28</td>
</tr>
<tr>
<td>Ear</td>
<td>2</td>
<td>67</td>
<td>196</td>
<td>118,400</td>
<td>226</td>
<td>58</td>
</tr>
<tr>
<td>Heart</td>
<td>4</td>
<td>90</td>
<td>110</td>
<td>122</td>
<td>111</td>
<td>1670</td>
</tr>
</tbody>
</table>

* Values expressed as mean cpm/mg tissue.
pouch was tested in the liquid scintillator, the activity measured in the millions of cPM/mg tissue. The fate of P32 injected into the cheek pouch after 3 weeks’ duration can be seen in Table II. The mean cPM/mg of tissue were low and were essentially the same for all lymphatic tissues regardless of time. The cheek pouch activity, in contrast, remained exceedingly high.

Thorotrast.—The results of this experiment are exemplified in Figs. 1 to 5, which contain a sample of x-rays of 1 animal taken over a period of time. X-rays of the other experimental animals were essentially the same. At the end of the 7th week the thorotrast was still concentrated within the cheek pouch. The x-ray photographs were cropped to highlight the cheek pouch. A full body view shows no radiopaque material localized in any of the viscera.

### Table II

<table>
<thead>
<tr>
<th>Days</th>
<th>No. of animals</th>
<th>Superficial cervical node</th>
<th>Right auricular node</th>
<th>Thymus</th>
<th>Spleen</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Right</td>
<td>Left</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>138 ± 55</td>
<td>169 ± 28</td>
<td>34 ± 16</td>
<td>145 ± 31</td>
<td>189 ± 50</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>32 ± 6</td>
<td>41 ± 12</td>
<td>53 ± 24</td>
<td>58 ± 4</td>
<td>8 ± 6</td>
</tr>
<tr>
<td>14</td>
<td>4</td>
<td>32 ± 15</td>
<td>32 ± 15</td>
<td>52 ± 21</td>
<td>27 ± 7</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>21</td>
<td>4</td>
<td>21 ± 6</td>
<td>22 ± 13</td>
<td>14 ± 6</td>
<td>15 ± 3</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

* Values expressed as mean cPM/mg tissue plus standard deviations.

What appears to be a large vessel in some of the figures is in essence merely a membranous fold of the everted pouch. No blood vessel (certainly no lymphatic vessel) of this diameter exists in the pouch of the hamster regardless of the animal’s size.

### DISCUSSION

It appears evident that the cheek pouch is without lymphatic vessels. The latter cannot be seen in routine histological preparations and the results reported here indicate that the classical techniques of ink injections or lymphangiograms likewise fail to demonstrate these vessels. Large molecules which leave the pouch must diffuse through the connective tissue of the membrane and pass into the lymphatics in the neck region which drain into the ipsilateral superficial cervical node. Occasionally one observes instances of cross-drainage into the axillary node or even into the contralateral cervical node. This pattern is not unique, for many mammals, including man, have variations in pathways plus similar arrangements of lymphatic vessels crossing the midline.

Our results are in agreement with those of Billingham et al. (1960) that the
areolar connective tissue is responsible for the privilege provided by the pouch. The data indicate that large molecules (chronic radiophosphate particles range from 200 to 20,000 millimicrons, thorotrast particles range from 102 to 515 millimicrons in diameters, and India ink particles, though small, 13 millimicrons, form agglomerates of macromolecular dimensions) are trapped or their passage is slowed down by the connective tissue of the pouch. The amount of P\textsuperscript{32} that escaped to the regional node was literally negligible when compared with the activity recorded in the regional node draining an area that has demonstrable lymphatics; e.g., auricular node. The time study with P\textsuperscript{32} also supports the "barrier" concept that no increase in radioactivity was uncovered in the regional nodes draining the pouch at the various intervals after the 1st day. The lower accumulations of P\textsuperscript{32} recorded after the 1st day could be explained by the more rapid diffusion of the smaller P\textsuperscript{32} molecules from the site of injection and by the temporary forcing of the radioactive material through the membrane during the injection. Lastly, the thorotrast x-rays illustrated that after 7 weeks the radiopaque material was still sealed within the membrane with no evidence that any of the original material had diffused out.

The problem that now arises is to correlate these data with other observations of transplantation in the hamster. For example, we reported (Shepro et al., 1960) that homologous tumor transplants in the cheek pouch evoked a cytological-histological response of the regional superficial cervical node that could only be interpreted as a "textbook" illustration of antibody or immunological activity. The nodes were hyperplastic, showing many germinal centers, plasmacytosis, thick medullary cords, sinuses filled with cells, and increased number of Scothorne's cortical "lymphoid" cells. How could this maximum histological response be congruent with the successful homotransplant, especially since the passage of large molecules (antigens) are inhibited by the cheek pouch membrane? Experiments are now in progress which possibly will explain whether the inconsistency is the result of some mechanism peculiar to the cheek pouch or if the histological picture of lymphatic tissue draining a graft, hitherto accepted as an important indicator of immunological activity, might be of less importance. Also under investigation is the fate of smaller antigens injected into the distal end of the cheek pouch.

**SUMMARY**

The cheek pouch of the hamster is alymphatic. Molecules, too large to penetrate the vascular endothelium, reach general circulation by slowly diffusing through the cheek pouch membrane to vessels found in the connective tissues in the neck and these vessels drain primarily into the superficial cervical node.
The tissues of the cheek pouch membrane limit the diffusion of large particles and it is this "barrier" which explains, in part, the "privilege" conferred by the cheek pouch.

We are indebted to Mr. Elmer Allard, R. T., Chief x-ray technician, Boston Veterans Hospital, for his technical assistance in photographing Figs. 1 to 5. We are equally indebted to Mr. Frederick W. Maynard for photographic assistance.

BIBLIOGRAPHY

EXPLANATION OF PLATE 41
Figs. 1 to 5. X-rays of 1 animal's everted cheek pouch injected with 0.05 ml of thorotrast taken at different time intervals. The pouch was everted, spread carefully, and pinned to a cork board with 3 to 6 bank pins. The board with the pouch was then taped to the x-ray table with surgical tape. Please note that in all x-rays the radiopaque material has not left the pouch. In the full body x-ray illustrations (which were cropped), no visible signs of the thorotrast appeared in any of the viscera. What appears to be a vessel in Figs. 2, 3, and 4 is an artefact resulting from a fold in the membrane when the pouch was pinned. The hamster cheek pouch does not contain a blood vessel or lymphatic vessel of this diameter.

**Fig. 1.** 1 hour postinjection. The faint whitish area to the left of the injection site is due to the surgical tape holding the pouch board in place. $\times 0.8$

**Fig. 2.** 1 day postinjection. The cheek pouch membrane was extended a little too far; hence the injected material does not appear as a concentrated mass. $\times 0.8$.

**Fig. 3.** 1 week postinjection. The radiopaqueness seen distal to the site of injection is due to the mechanics of spreading the pouch membrane and not to a diffusion of the thorotrast. $\times 0.8$.

**Fig. 4.** 2 weeks' postinjection. $\times 0.6$.

**Fig. 5.** 7 weeks' postinjection. $\times 0.6$. 
(Shepro et al.: Cheek pouch and transplantation immunity)