Enhancement of Growth of Chick Host Spleens following Chorio-allantoic Membrane Grafts of Homologous Tissues

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WITH ONE PLATE

INTRODUCTION

The phenomenon of enlargement of the host chick embryo spleen, following grafts of homologous adult chicken tissues to the chorio-allantoic membrane (CAM), affords the investigator an excellent tool for the study of growth.

Initial observations of this phenomenon were made by Danchakoff (1916) and Murphy (1916). Grafts of adult chicken spleen on the chorio-allantoic membrane of 9-day-old chick embryos brought about a striking enlargement of the host spleens after 8 additional days of incubation. The phenomenon was later studied by Ebert (1951), who showed that the effect was only partially organ-specific. Grafts of thymus and liver affected the weight of the host spleen, but in each case the effect was far smaller than that observed with splenic transplants. Andres (1955) found that injected kidney and liver debris, which elicited an increased mitotic index in the homologous host organ, was not inhibited in its action by killing the cells through freezing and subsequent thawing. Van Haeften (1958) recently reported hypertrophy of the embryonic host spleen following the administration of cell-free homogenates of spleen to the CAM.

The above findings may be interpreted as being in accord with earlier template-antitemplate hypotheses (Weiss, 1947; Tyler, 1947): the introduced template, supplementing existing cellular templates, catalyse the formation of additional antitemplates leading to the enlargement of the homologous organ. Intact and proliferating cells as such, were sufficient but not necessary to produce the enlargement.

Ebert (1954), using adult chicken spleen and kidney tissues labelled with $^{35}S$,
observed a selective incorporation of radioactivity into the proteins of homologous tissues of the host chick embryos. He envisaged a transfer of 'tissue specific protein constituents larger than amino-acids' from the grafts into proteins of the host tissue, and thus strongly suggested a 'building block' mechanism rather than a template or catalytic mechanism.

More recently, Ebert (1957, 1958 a, b, 1959) demonstrated that radioactively labelled (S\textsuperscript{35} methionine) nonviable homogenates of organs injected into the vascular system of the 9- or 10-day chick embryo were differentially incorporated in the homologous organ. Examination of nuclear, mitochondrial, microsomal, and supernatant fractions of the labelled homogenates showed clearly a localization of only the microsomal and supernatant fractions, and not of the nuclear and mitochondrial fractions. Ebert emphasized, however, that these experiments 'deal only with one aspect of the problem, namely predominant localization'.

In 1951 Ebert reported that the ability to evoke enlargement of the host embryo spleen was correlated with an increase in the number of antigens in the developing donor spleen. Grafts from embryonic chick donors of less than 14 days' incubation did not elicit enlargement of the homologous organ of the host. However, grafts from older embryonic donors produced a significant increase in the weight of the host spleen.

Recently, Simonsen (1957) presented evidence which suggested that intact donor cells capable of proliferation and antibody production were necessary in this phenomenon of host spleen enlargement. He observed an enlargement of the host spleen following injections of suspensions of chicken spleen cells or whole adult chicken blood into chick embryos on the 18th day of incubation. When similar experiments were performed on inbred lines of mice, the host spleen was not enlarged following the injection of cells from donors of the same strain. This observation was also made later in the chicken (Cock & Simonsen, 1958).

Cock & Simonsen (1958) postulated that the injected donor cells proliferate, colonize the reticulo-endothelial system of the host, and, responding to the antigenic stimulus presented them by the host, proceed to attack the host cells 'both by producing antibodies against the host erythrocytes, and by means of a homograft reaction'. Simonsen (1957) had previously found, 'by means of Coombs' test', antibodies against blood group antigens of the recipient in the blood of the recipient.

DeLanney and Ebert (see Ebert, 1958 a, b, and DeLanney, 1958) have also presented striking evidence of the graft-versus-host reaction, namely, destruction of the homologous organ of the host and subsequently the host embryo itself, following grafts of immunologically competent adult tissues. Whether the grafts were made to the chorio-allantoic membrane or to the coelom of the chick, or to the coelom or dorsal fin of the salamander, the basic results have been consistent with the hypothesis of a graft-versus-host reaction. Ebert (1958 a, b) has made it clear that although the occurrence of the graft-versus-host reaction does not necessarily argue against the hypothesis of organ-specific growth regulation, it
does make tests of the latter hypothesis difficult when immunologically competent tissues are employed.

The original objective of the present investigation was to determine whether the antigen-antibody mechanism was involved in this enlargement of the host embryo spleen following CAM transplant of adult chicken spleen tissue. The cells of the donor were inactivated, i.e. rendered incapable of mitosis, by freezing and subsequent thawing, and by X-irradiation of the tissues prior to implantation on the CAM. In a second series of experiments, the activity of the donor spleen tissue when implanted on hosts from the same inbred line, was compared with its activity on hosts from a different inbred line of chickens. In a third series of experiments an attempt was made to increase the activity of the adult donor spleen tissue (1) by pre-injection with embryo spleen tissues, and (2) by the implantation of spleens from more distantly related animals, e.g. the turkey and the pheasant.

METHODS AND MATERIALS

'Synthetic' dominant white and White Leghorn chickens and eggs were used in this investigation. Eggs were incubated for 9 days at 99.5° F.

The method of transplantation of tissues to the chorio-allantoic membrane (CAM) adopted in this study was essentially that described by Hamburger (1942). A quadrilateral window (1 x 1 cm.) was cut in the shell with a hack-saw blade. The shell membrane was punctured and reflected, and a piece of tissue approximately 1 x 1 x 2 mm. (weight 5 to 10 mg.) was placed on the CAM. The shell membrane and the piece of cut shell were replaced and covered with a plastic tape. The edges of the tape were sealed with paraffin wax. The eggs were placed in the incubator with the small end down.

The operated eggs were incubated for 8 additional days. On the 17th day of incubation the grafts were examined and each graft was graded, independently of the size of the host spleen, according to its condition as follows:

1. Fully successful: graft well vascularized, pinkish, and as large as, or larger than, the original piece of grafted tissue.
2. Partially successful: graft pink but smaller than the original graft.
3. Not successful: graft brown or green, apparently without blood-vessels coursing through it. The darkened and somewhat shrunken piece of tissue can be readily dislodged from the CAM.

The spleens of the embryos were then removed and washed in saline. After excess moisture was removed, the spleens were weighed to the nearest 0.2 mg. on a Roller–Smith torsion balance.

The findings reported in these studies are based on 33 experiments involving more than 3,000 embryos.

Each experiment included unoperated controls and embryos in which 0.05 to 0.1 c.c. of chick Ringer's solution was dropped on the chorio-allantoic membrane as well as embryos which received grafts. Because adult chicken kidney
tissue did not significantly affect the size of the host spleen, such grafts frequently also served as controls. The eggs were coded and subjected to the various treatments in a random sequence.

RESULTS

Experiment 1: The effects of frozen spleen grafts on the homologous host organ

Fragments of fresh adult chicken spleen, liver, and kidney tissue were frozen in 15 to 30 seconds by placing them on the holder of a CO₂ freezing microtome and permitting the gas to escape rapidly. Tissues were also frozen by placing them in a freezer (—20° C.) for 1 to 5 days. Prior to implantation, the tissues were thawed at room temperature, and were either cut into small pieces (1 x 1 x 2 mm.) or macerated with an equal amount of saline in a conical test tube with a fitted and ground glass rod. Microscopical examination of the macerated tissue showed intact cells, both singly and in clumps. In some cases, fresh adult chicken spleen was heated in a water bath at 56° C. for an hour before implantation.

Table 1

Effect of frozen and heated adult chicken tissue on the weight of the chick embryo spleen

<table>
<thead>
<tr>
<th>Donor tissue</th>
<th>Fully successful</th>
<th>Partially successful</th>
<th>Not successful</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage</td>
<td>Av. wt. of host spleen</td>
<td>Percentage</td>
</tr>
<tr>
<td>Fresh spleen</td>
<td>79 (129)*</td>
<td>26-8</td>
<td>13 (22)</td>
</tr>
<tr>
<td>Frozen spleen</td>
<td>13 (18)†</td>
<td>10-7</td>
<td>38 (53)</td>
</tr>
<tr>
<td>Heated spleen</td>
<td>0 (0)</td>
<td>.</td>
<td>13 (1)</td>
</tr>
<tr>
<td>Fresh liver</td>
<td>20 (2)</td>
<td>42-3</td>
<td>40 (4)</td>
</tr>
<tr>
<td>Frozen liver</td>
<td>32 (8)</td>
<td>8-7</td>
<td>44 (11)</td>
</tr>
<tr>
<td>Fresh kidney</td>
<td>57 (33)</td>
<td>14-3</td>
<td>21 (12)</td>
</tr>
<tr>
<td>Frozen kidney</td>
<td>9 (3)</td>
<td>15-3</td>
<td>48 (16)</td>
</tr>
<tr>
<td>Not operated controls</td>
<td>(81)</td>
<td>9-2</td>
<td>.</td>
</tr>
<tr>
<td>Saline controls</td>
<td>(71)</td>
<td>11-7</td>
<td>.</td>
</tr>
</tbody>
</table>

* Figures in parentheses indicate No. cases.
† Refer to experiment 1 in text.

Macerated or crushed fresh (unfrozen and unheated) spleen (5 to 10 mg. of tissue) gave a higher percentage of successful incorporation on the CAM than frozen or heated spleen—as the presence, usually, of 2 or 3 large nodules on the CAM demonstrated. In these cases the host spleens also tended to be more uniform in size and appearance. The frozen and heated tissues, both whole and macerated, when separated from the CAM were soft and crumbly, and white or yellowish in colour. The host spleen size was not significantly enhanced, even in those cases in which the grafts may have been considered ‘successful’ (Table 1). These ‘successful’ grafts appeared to be well vascularized and were as large as
the original pieces of implanted tissue. However, sections of these grafts showed large areas of necrosis and no detectable mitotic figures.

These findings are supported fully by a similar analysis of over 400 frozen and alcohol-fixed spleen grafts by Ebert (personal communication).

However, in view of the low percentage of fully successful ‘takes’, one cannot yet conclude that the frozen and heated donor spleen tissue cannot influence the size of the host spleens. The inability to stimulate the host organ may have been due simply to the failure to ‘take’.

Although fresh chicken liver tissue stimulated growth of the host spleen, frozen liver tissue did not. Fresh and frozen kidney tissue did not stimulate the host spleen to a significant extent.

Experiment 2: The effects of X-irradiated spleen grafts on the homologous host organ

Because a majority of the frozen spleen tissues failed to ‘take’ successfully on the CAM, another method was sought which would destroy the ability of the cells to proliferate without reducing the ability of the tissues to ‘take’ successfully on the CAM.

X-irradiation of the adult spleen tissue before implantation was tried because it is well recognized that at high doses it can block or inhibit mitosis. Such an approach should, therefore, be useful in demonstrating whether donor cells capable of undergoing mitosis are necessary for host spleen enlargement.

Accordingly, fragments of adult chicken spleen and kidney tissues, approximately $10 \times 5 \times 2 \text{ mm.}$, were irradiated for 5 to 60 minutes with a therapeutic-type X-ray machine, operating at 250 kV and 15 mA. A 0.5 mm. Cu filter was used, giving a filter factor (HVL) of 1.38 mm. Cu. The tissues were irradiated at a rate of 41 r/min., as measured with a Victoreen r meter, and at a distance of 50 cm. from the source. The tissues were kept below 10°C. in a water bath during handling and X-irradiation to reduce autolysis during the long period of exposure.

After irradiation, the tissues were cut into smaller pieces and implanted on the CAM. A specimen of each irradiated tissue was fixed in Baker’s calcium formol or Bouin’s fixative for further histological study.

The effect of X-irradiation on the ability of the tissues to stimulate growth of the host spleen is summarized in Table 2.

These results indicate that:

1. At lower doses (between 205 and 820 r) the X-irradiated adult chicken spleen stimulated the growth of the host chick spleen. Microscopical examination of sections of well incorporated grafts showed many normal appearing spleen cells. Mitotic figures ranged between 3 and 5 per cent. (Plate, figs. 1, 2).

2. At higher doses (between 1,230 r and 1,845 r), a significant decrease was observed in the ability of the donor tissue to influence the size of the host spleen. Microscopical examination of sections of successfully incorporated grafts
showed a few well-stained and apparently normal spleen cells. Mitotic figures, however, were relatively rare, i.e. between 0 and 1 per cent. (Plate, figs. 3, 4).

3. At doses above 1,845 r, no stimulation of the host spleen was observed. Many of the X-irradiated grafts were embedded in, or enclosed by, the chorio-allantoic membrane. The majority of the grafts were necrotic. Microscopical examination of sections of the irradiated grafts showed many dark, small, pyknotic nuclei (Plate, figs. 5, 6). The percentage of successful grafts was decreased.

**Table 2**

*Effect of X-irradiated chicken spleen and kidney tissue on the homologous host chick organ*

<table>
<thead>
<tr>
<th>Donor</th>
<th>Dose (r)</th>
<th>Percentage</th>
<th>Number of cases</th>
<th>Average weight of host spleen (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>0</td>
<td>70</td>
<td>48</td>
<td>37.5</td>
</tr>
<tr>
<td></td>
<td>205</td>
<td>67</td>
<td>16</td>
<td>32.5</td>
</tr>
<tr>
<td></td>
<td>410</td>
<td>80</td>
<td>37</td>
<td>25.2</td>
</tr>
<tr>
<td></td>
<td>820</td>
<td>75</td>
<td>27</td>
<td>25.2</td>
</tr>
<tr>
<td></td>
<td>1,230</td>
<td>65</td>
<td>22</td>
<td>18.1</td>
</tr>
<tr>
<td></td>
<td>1,845</td>
<td>46</td>
<td>5</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>2,050</td>
<td>50</td>
<td>5</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>2,460</td>
<td>15</td>
<td>6</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>0 and 2,460*</td>
<td>69</td>
<td>11</td>
<td>28.54</td>
</tr>
<tr>
<td>Kidney</td>
<td>0</td>
<td>59</td>
<td>28</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>1,845</td>
<td>47</td>
<td>22</td>
<td>10.7</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>.</td>
<td>84</td>
<td>9.6</td>
</tr>
</tbody>
</table>

* Two grafts transplanted concomitantly to the same host.

This experiment shows that adult chicken spleens, subjected to high doses of X-irradiation (1,230 r to 1,845 r) prior to transplanting to the CAM, lose the capacity to increase the weight of host spleens, even though they are well incorporated as grafts and appear to be normal. Grafts of 'heavily' irradiated (2,460 r) spleen and kidney tissue did not result in spleen weights which were less than that of normal or saline controls. Moreover, concomitant implantation of both non-irradiated and irradiated (2,460 r) spleen brought about the characteristic increase in the size of host spleens.

The ability to stimulate growth of the host spleen is thus apparently correlated with the number of mitotic figures observed in sections of the X-irradiated graft. It is well recognized that cellular proliferation is important in antibody formation (Fagraeus, 1948; Harris, Harris, Beale, & Smith, 1954), and that X-irradiation can depress or inhibit antibody formation (Taliaferro & Taliaferro, 1951; Hale & Stoner, 1954). These observations suggest, therefore, that the antibody-forming mechanism may be involved in host spleen enlargement. However, a direct test for the ability of the X-irradiated tissue to produce antibody was not conducted.
Experiment 3: Comparison of growth stimulating effects of spleens from two inbred lines of chickens

The previous experiment demonstrates that X-irradiation of adult chicken spleen tissue can destroy its ability to stimulate growth of embryonic spleen. Many workers (e.g. Hale & Stoner, 1954) have reported that X-irradiation can destroy antibody formation. Accordingly, a study was made of the possible role of immunological mechanisms in this reaction.

Adult spleens from two inbred lines of White Leghorn chickens, selected for leukemia susceptibility (S) and leukemia resistance (R) were implanted, reciprocally and within lines, on the CAM of embryos from these two lines. If immunological mechanisms are involved, one would expect a greater reaction between the two lines than within each line. Although the two lines were not exactly of the same degree of inbreeding, both have been maintained, with a limited number of matings and without introduction of outside stock, for over 20 years. The experiments were repeated three times, with essentially similar results.

Table 3

Effect of donor spleens from two inbred lines of chickens on the spleen of the host chick embryo

<table>
<thead>
<tr>
<th>Adult donor</th>
<th>Egg host</th>
<th>Number of cases</th>
<th>Average weight of host spleen (mg.)</th>
<th>S.E. of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>S*</td>
<td>S</td>
<td>18</td>
<td>26.4</td>
<td>3.346</td>
</tr>
<tr>
<td>R†</td>
<td>S</td>
<td>23</td>
<td>18.6</td>
<td>1.991</td>
</tr>
<tr>
<td>Saline</td>
<td>S</td>
<td>27</td>
<td>10.1</td>
<td>0.381</td>
</tr>
<tr>
<td>S</td>
<td>R</td>
<td>46</td>
<td>45.5</td>
<td>3.648</td>
</tr>
<tr>
<td>R</td>
<td>R</td>
<td>28</td>
<td>13.45</td>
<td>1.069</td>
</tr>
<tr>
<td>Saline</td>
<td>R</td>
<td>52</td>
<td>9.88</td>
<td>0.333</td>
</tr>
<tr>
<td>S</td>
<td>S×R</td>
<td>13</td>
<td>47.4</td>
<td>5.85</td>
</tr>
<tr>
<td>R</td>
<td>S×R</td>
<td>9</td>
<td>16.0</td>
<td>1.96</td>
</tr>
<tr>
<td>Saline</td>
<td>S×R</td>
<td>12</td>
<td>9.4</td>
<td>2.94</td>
</tr>
</tbody>
</table>

* S line selected for leukemia susceptibility.
† R line selected for leukemia resistance.

Spleens from adults of the S line brought about a lesser stimulation of the spleens in embryos from the S line than in embryos from the R line. Similarly, spleens from the R lines brought about a lesser stimulation in R embryos than in S embryos. On the basis of a pooled t test, the differences were significant at the 1 per cent. and 5 per cent. levels respectively (Table 3).

When spleens from adults of the S line were implanted on the CAM of the F₁ (R×S) embryos, an enlargement of the host spleen was observed which was similar in magnitude to that obtained following grafts of S line spleens on R line embryos. Spleens from R line adults, when implanted on the CAM of the F₁
SPLENIC ENLARGEMENT IN CHICK EMBRYOS

(R x S) embryos, did not produce as great an enlargement as those on S line embryos. This experiment was not repeated, due to the unavailability of material.

It was further noted that spleens from adult S line chickens consistently brought about a correspondingly greater increase in the spleen of the host (regardless of the latter's origin) than spleens from adult R line chickens. Similar effects were noted in this laboratory in 1957 (unpublished) when the growth-stimulating effect of spleens from the same S line was compared with that produced by spleens from a non-inbred flock of White Leghorn chickens.

The two lines used were not checked for 'effective homozygosity' by skin grafting. However, the two lines are apparently sufficiently different to respond characteristically and consistently to the different treatments.

These results are essentially in agreement with those of Cock & Simonsen (1958), who used two highly inbred White Leghorn lines. In their study, gross enlargement of the spleen and liver resulted when blood from adult birds of an inbred line (I) was injected into newly hatched chicks of a cross between two highly inbred lines (C and I). On the other hand, Fi blood injected into Fi chicks, and either I or Fi blood injected into I chicks, produced only a relatively slight enlargement of the spleen and liver.

Cock & Simonsen concluded that the observed enlargement was due to the multiplication of donor cells within the host and the launching by them of an immunological attack against the host. Simonsen (1957) had previously demonstrated the presence of antibodies against blood group antigens of the host in the blood of the host.

Results of the present study (experiment 3) suggest that an antigen-antibody type mechanism may be involved. This led to the question: could this reaction be increased (1) by exposing the adult chicken donor to the chick embryo spleen (thus stimulating antibody production) prior to implantation on the CAM or (2) by the grafting of spleens from such animals as the pheasant, turkey, rat, or guinea-pig (which would be expected to differ antigenically from the host chicken embryo)?

**Experiment 4: The effect of spleens from adult chickens injected with chick embryo spleen prior to implantation on the CAM**

To test the first possibility mentioned above seven adult White Leghorn and 'synthetic' dominant white hens were injected with homogenized frozen and fresh spleens from embryos incubated for 15–19 days. At least 10 injections, over a period of not more than two months, were given to each animal. Approximately 10 to 15 mg. of tissue were injected into each animal intravenously or intra-peritoneally. The animals were bled and killed one week after the last injection.

As shown in Table 4, spleens from chickens injected with chick embryo spleens did not produce a greater increase in the size of the host embryo spleen.
Instead, the stimulating effect of spleen from the injected chickens was consistently and significantly less (at the 1 per cent. level, using pooled *t* test) than that of spleens from non-injected chickens.

### Table 4

*Effect of spleen from donors injected with 15- to 19-day chick embryos*

<table>
<thead>
<tr>
<th>Donor</th>
<th>Number of cases</th>
<th>Average weight of host spleen (mg.)</th>
<th>S.E. of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-injected</td>
<td>78</td>
<td>33.08</td>
<td>3.034</td>
</tr>
<tr>
<td>Injected</td>
<td>97</td>
<td>24.21</td>
<td>1.288</td>
</tr>
<tr>
<td>Saline</td>
<td>56</td>
<td>9.53</td>
<td>0.247</td>
</tr>
</tbody>
</table>

If circulating antibodies to the injected antigens are involved in the stimulation, one would have expected a greater response from the injected donor spleens than that obtained from the non-injected donors. This was not the case.

However, the titres of the antisera, obtained in this laboratory by interfacial 'ring' tests, were low or negligible (1:4). The low titres of these antisera may have been due to the weak antigenicity of the chick embryo spleen, as well as to the antigenic composition of the chick spleen, which may be similar to that of the adult chicken. Ebert (1951) reported that by the 18th day of incubation all antigenic components present in the adult can be found in the embryo.

This problem is being further investigated in this laboratory by using antisera prepared in one line of White Leghorn chickens against the spleen from a second inbred line, combined with complement according to a procedure described earlier (Mun, 1958). It is possible that the injected homogenates of the embryos fail to supply antigens to the adult chicken in the same form in which they are made available to the donor graft by the host embryo.

### Experiment 5: Effect of spleens from different animal species on the growth of the chick embryo spleen

To test the possibility that the hypertrophy of host spleens might be significantly increased by the use of *heterologous* donor material, spleens from pheasants, turkeys, guinea-pigs, and rats were implanted on the 9-day-old chick embryo CAM as described above.

The turkey and pheasant spleen grafts were incorporated in the chorioallantoic membrane. In some instances the grafts were more than 2 to 3 times the size of the original piece of implanted tissue. The turkey and pheasant spleen tissues affected the size of the chick host spleen to a significant degree (0.1 per cent. and 1 per cent. levels, respectively). However, these effects were not greater but significantly less (at the 5 per cent. and 1 per cent. levels, respectively) than that of adult chicken spleen grafts on the homologous host organ (Table 5).
Both rat and guinea-pig spleen grafts, on the other hand, did not 'take' well and, probably for this reason, did not influence the size of the host spleen. The reason for this failure to 'take' is not known.

**TABLE 5**

*Effect of spleen from different animals on the weight of the chick embryo spleen*

<table>
<thead>
<tr>
<th>Animal</th>
<th>Percentage</th>
<th>Number of cases</th>
<th>Average weight of host spleen (mg.)</th>
<th>S.E. of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>67</td>
<td>41</td>
<td>26.0</td>
<td>2.434</td>
</tr>
<tr>
<td>Turkey</td>
<td>79</td>
<td>19</td>
<td>16.6</td>
<td>1.525</td>
</tr>
<tr>
<td>Pheasant</td>
<td>86</td>
<td>17</td>
<td>12.8</td>
<td>1.582</td>
</tr>
<tr>
<td>Rat</td>
<td>6</td>
<td>8</td>
<td>12.2</td>
<td>..</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>5</td>
<td>8</td>
<td>11.2</td>
<td>..</td>
</tr>
<tr>
<td>Saline</td>
<td>..</td>
<td>46</td>
<td>9.14</td>
<td>0.328</td>
</tr>
</tbody>
</table>

The existence of a natural antibody in the rejection of mouse tumour-cells by the chick embryo has been reported by Green & Lorincz (1957). They showed that the death of the injected tumour-cells and the appearance in the chick embryo of serum gamma globulins occurred on the 17th day of incubation. In our study, however, the tissues were implanted on the 9th day of incubation and removed on the 17th day, and it is therefore unlikely that the failure of the rat and guinea-pig spleen graft to 'take' was due to a natural immunity.

Thus, although the grafts of turkey and pheasant spleen were well incorporated on the CAM, the host chick spleen was only slightly enlarged. As has already been stated, in view of the greater genetic diversity between the turkey and the chick host, a greater stimulation of growth might have been expected if the host spleen enlargement is due to an antigen-antibody type reaction.

**DISCUSSION**

The available data suggest that the phenomenon of growth stimulation of the host chick embryo spleen by homologous adult organ tissues involves at least two steps: (1) the initial stimulation leading to increased mitotic index and a subsequent enlargement of the host organ, (2) death of the host following debilitation and subsequent atrophy of the spleen and hematopoietic organs. The first step has been studied extensively by the students of growth; the second was unexpectedly encountered by workers in embryology and radiation protection. Although there may be common basic mechanisms underlying the two, each step may have its own peculiar mechanism.

A diffusible substance, resistant to the effects of freezing and subsequent thawing, may be involved in the first step. This was clearly demonstrated in the experiments of Andres (1955) where an increased mitotic index in the liver and kidney was observed following injection of frozen and thawed non-viable
homologous organ debris. The work of Ebert (1958 a, b) suggests that the growth stimulating substance(s) may be identified with the microsomal and supernatant fractions of radioactively labelled (S\textsuperscript{35} methionine) non-viable homogenates of organs. Continuous stimulation by substances of this sort may eventually bring about an increase in the size of the spleen, although this has not been actually demonstrated.

The failure of the frozen spleen material to stimulate growth of the host spleen (experiment 1) does not contradict this possibility of a diffusible growth stimulating factor or factors. The failure to stimulate may be due to the failure to 'take' and thereby supply the host spleen with a continuous flow of stimulating substances. Van Haeften (1958), using larger amounts of cell-free spleen homogenates (200 to 500 mg. of spleen homogenate) was able to observe an enlargement of the host spleen. If corroborated by subsequent work, his report is of profound interest, but it must be noted that the criteria for the absence of intact cells in the homogenates are not altogether clear; the use of filters is not reported.

In view of this report that spleen enlargement can be brought about in the absence of intact donor cells, it is difficult to picture the resulting enlargement as being exclusively due to a massive migration of intact cells from the donor to the host.

In a study employing radioactive tracers, Ebert (1954) presented three lines of evidence militating against this possibility:

(1) Microscopic examination of histologic preparations of embryonic host kidney failed to reveal any accumulation of cells which was not incorporated into the normal cellular pattern of the kidney.

(2) A study of autoradiograms of grafts of kidney, spleen, and other tissues of host embryos indicated that while there was, in general, a higher level of radio-sulphur in the homologous tissues, there was no localization in any region or in any specific cell type of the kidney or spleen.

(3) Analysis of DNA in enlarged and normal spleens showed that the DNA content of the enlarged spleens did not increase concomitantly with the increase in protein content.

The hypotheses of Weiss (1947) and Tyler (1947) have been frequently invoked in the interpretations of these events. However, the possibility of differentiation and rapid proliferation of a 'seed' population of donor cells in the specialized host environment cannot be discounted.

It appears, at first glance, that the observations of Cock & Simonsen (1958) on highly inbred lines, and the results in experiment 3 of this study, cannot be adequately explained in terms of a template-antitemplate mechanism. However, it must be noted that even in the use of highly inbred material, such as was available to Cock & Simonsen (1958), there was a slight enlargement of the host spleen when blood from pure I adult fowls was injected into I chicks. In experiment 3 of our studies, when S and R adult spleens were grafted on embryos from the same line, there was also a slight but significant increase in size of the spleen.
Thus, the hypotheses of Weiss and Tyler cannot be completely discounted. This slight increase, in our case, may be due to antigenic differences between the two lines.

The severe anaemia, local reactions at the site of injection, and death of the host chick observed by Cock & Simonsen (1958) can very well be explained by a graft-versus-host-type of reaction. This step, however, is probably secondary and may be considered as separate from the first 'stimulation' step described above. The second step requires intact cells (Simonsen, 1957).

In our studies a small proportion of the embryos receiving CAM grafts of adult chicken spleen tissue were apparently afflicted with an immune reaction. Approximately 10 per cent. of the enlarged spleens appeared mottled and showed areas of necrosis in section. A similar proportion of the chicks receiving grafts of adult chicken spleen on the 9th day of incubation were small or stunted (approximately 50 per cent. of body-weight of the controls at 4 to 6 weeks' post-hatching). However, the bulk of the enlarged spleens and hatched chicks appeared normal. The role of haemorrhage, infection, and rate of healing associated with the CAM grafting technique remains to be examined.

In our attempt to discover how the grafted donor spleen tissue affects the host spleen we observed that the reaction is not aggravated by methods which are supposed to increase the amount of circulating antibodies against the host spleen or to incite a greater immunologic reaction. On the contrary, the response is in the opposite direction. Thus, although it appears that the host must be challenged by immunologically competent tissues, the mechanism involved in the subsequent enlargement is probably not dependent on antibody activity. This interpretation is, of course, based primarily on evidence obtained by the use of the CAM transplant technique. Indeed, the failure to obtain the expected response in experiment 5 may be due to the inability of the donor cells to colonize the host spleen in sufficient numbers. Experiments are under way in this laboratory to test this possibility, viz., the injection of homogenate of turkey spleen into the chick embryo.

**SUMMARY**

1. A striking enlargement of the host chick embryo spleen was obtained following chorio-allantoic grafts of adult chicken spleen tissue.
2. When the adult chicken spleen tissue was killed by freezing and thawing before implantation on the chorio-allantoic membrane, no enlargement of the host chick embryo spleen was observed. However, a large number of the frozen grafts failed to 'take'.
3. X-irradiation of the donor spleen, at a level sufficient to block or inhibit mitosis, also removed the growth-stimulating ability of the grafted tissue in spite of the fact that the grafts were well incorporated. This suggested that donor cells capable of undergoing mitosis were necessary for producing enlarged host spleens.
4. Because of the possibility that antibody-forming mechanisms were destroyed at these levels of irradiation, the involvement of such mechanisms in this reaction was studied.

5. In a comparison of the effect of spleen grafts between two inbred lines of White Leghorn chickens (S and R), a lesser stimulation was obtained when spleens from S line chickens were implanted on S line embryos than on R line embryos. Similarly, a lesser stimulation was obtained when R spleens were implanted on R embryos than on S embryos. Spleens from S line chickens also evoked a greater response than spleens from R line chickens.

6. To test the role of antibodies in this reaction, spleens from adult chickens previously injected with chick embryo spleens were implanted on the chorio-allantoic membrane. In all cases (7) the spleens from the injected chickens stimulated the host embryo spleen to a significantly lesser degree than spleens from non-injected chickens.

7. Spleens from different animal species, including the turkey, pheasant, rat, and guinea-pig, were implanted on the chorio-allantoic membrane of the early chick embryo. The turkey and pheasant spleens stimulated the host chick embryo spleens to a significantly lesser degree than adult chicken spleen in spite of the greater genetic diversity and subsequently successful incorporation in the chorio-allantoic membrane.

8. From the available evidence the authors suggest that the enlargement of the host chick embryo spleen following chorio-allantoic membrane grafts of homologous adult chicken tissue probably involves two separate steps: (1) a stimulation which may occur in the absence of intact cells; (2) an immune-type reaction which results in necrosis of areas in the host spleen, and stunting of growth of the hatched chicks.

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REFERENCES

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**EXPLANATION OF PLATE**

Fig. 1. Section of well-incorporated graft of non-irradiated adult chicken spleen. ×60.

Fig. 2. Section of above graft at higher magnification showing mitotic figures. ×950.

Fig. 3. Section of well-incorporated graft of adult chicken spleen irradiated for 30 minutes (1,230 r) prior to implantation. ×60.

Fig. 4. Section of above graft at higher magnification. ×950.

Fig. 5. Section of well-incorporated graft of adult chicken spleen irradiated for 1 hour (2,460 r) prior to implantation. ×60.

Fig. 6. Section of above graft at higher magnification. ×950.

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