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# STUDIES ON THE ONTOGENY OF THE MOUSE IMMUNE SYSTEM I. Cell-Bound Immunity

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# STUDIES ON THE ONTOGENY OF THE MOUSE IMMUNE SYSTEM. I. CELL-BOUND IMMUNITY

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ABSTRACT

The presence of potential immunologically competent cells in the various tissues of embryonic, newborn and adult mice was demonstrated by means of a modified 'graft-vs-host' method and by the injection of chromosomally marked cells into irradiated recipients. The results indicate that lymphoid stem cells which have the potential to participate in cell-bound immune reactions appear in the placenta and liver by the 9th or 10th day of gestation. Throughout pregnancy they are found in the liver. During the 11th to 14th days of gestation these stem cells are present in the upper trunk or in the thymus. On about the 15th day they appear in the lung and toward the end of the pregnancy in the fetal bone marrow and spleen. There was little or no evidence of the presence of lymphoid precursors capable of maturation to cells which mediate cell-bound immune responses in the gut prior to birth.

Following parturition lymphoid stem cells were present in the liver, Peyer's patches, lung, bone marrow, lymph nodes, spleen, blood and thymus. However, by six weeks after birth the bone marrow appeared to be the major or sole source of these cells.

Certain of the data suggest that immunoglobulin producing cells (as opposed to those which mediate cell-bound immunity) may arise in the yolk sac on the 9th or 10th day of gestation. Further, these immunoglobulin producing cells may reside in the gut as a relatively pure cell population after the 16th day of gestation.

## SUMMARY

The Problem:

The origin and development of the immune system of the mammalian embryo has great relevance to the field of tissue homotransplantation. Previous work from this Laboratory has shown that lymphoid stem cells are derived from liver and other nonthymic tissues during embryonic life and has suggested that the functional maturation and/or proliferation of these cells is dependent upon their residence in thymic tissue. The present experiments were designed to examine more critically the origin and distribution of those stem cells which have the potential to participate in 'delayed hypersensitivity' or cell-bound immune reactions.

The Findings:

The presence of potential immunologically competent cells in the various tissues of embryonic, newborn and adult mice was demonstrated by means of a modified 'graft-vs-host' method and by the injection of chromosomally marked cells into irradiated recipients. The results indicate that lymphoid stem cells which have the potential to participate in cell-bound immune reactions appear in the placenta and liver by the 9th or 10th day of gestation. Throughout pregnancy they are found in the liver. During the 11th to 14th days of gestation these stem cells are present in the upper trunk or in the thymus. On about the 15th day they appear in the lung and toward the end of the pregnancy in the fetal bone marrow and spleen. There was little or no evidence of the presence of lymphoid precursors capable of maturation to cells which mediate cell-bound immune responses in the gut prior to birth.

Following parturition lymphoid stem cells were present in the liver, Peyer's patches, lung, bone marrow, lymph nodes, spleen, blood and thymus. However, by six weeks after birth the bone marrow appeared to be the major or sole source of these cells.

Certain of the data suggest that immunoglobulin producing cells (as opposed to those which mediate cell-bound immunity) may arise in the yolk sac on the 9th or 10th day of gestation. Further, these immunoglobulin producing cells may reside in the gut as a relatively pure cell population after the 16th day of gestation.

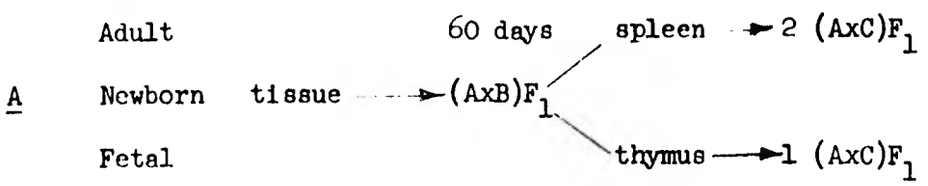
## INTRODUCTION

Previous work has shown that mouse fetal liver and perhaps other nonthymic embryonic tissues give rise to potential immunologically competent cells prior to and after the appearance of the thymic rudiment (1,2), and that in the adult mouse the bone marrow serves as the major or perhaps as the sole source of these stem cells (3). Further, it has been demonstrated that lymphoid stem cells are dependent upon the thymus for their ability to participate in graft-vs-host reactions (4-6) or to produce specific antibody in response to antigenic stimulation (7). The present experiments were designed to examine more critically the origin and distribution of those stem cells which have the potential to participate in 'delayed hypersensitivity' or cell-bound immune reactions. A later communication will present data with regard to the ontogenesis of immunoglobulin producing cells.

## MATERIALS AND METHODS

The presence of immature lymphoid stem cells was demonstrated by a modified parental-$F_1$ hybrid, 'graft-versus-host', method. When lymphoid tissues from a homozygous adult donor are injected into sublethally irradiated $F_1$ hybrids, one parental strain of which is identical to that of the donor, deaths will occur as a result of an immunological reaction by the donor cells against the transplantation antigens of the second parent of the hybrid. Survivors can be obtained, however, by transplanting small numbers of donor cells. If it is postulated that mature immunologically competent cells are consumed during the course of a graft-versus-host reaction (8), then surviving mice which had been injected with mature cells only should no longer contain lymphoid cells of donor origin (3). On the other hand, immature lymphoid cells are demonstrably incapable of responding to the foreign antigens of the host and survive in significant numbers (3,5,6). If after 60 days the lymphoid tissues of this primary host are injected into a second $F_1$ hybrid (one parental strain identical to that of the donor of the injected tissue but the second parental strain differing from the second parent of the primary host) deaths will occur as a result of the maturation of the injected lymphoid stem cells in the primary host (4). On this basis, dissociated cells from various tissues of homozygous fetal, newborn or adult mice were injected into sublethally irradiated $F_1$ hybrids. Sixty days later the survivors were killed individually. The spleen and lymph nodes and the thymus were gently disrupted separately; the resultant cell suspensions were injected into one (thymus) or two (spleen and lymph nodes) sublethally irradiated secondary recipients. The death of these secondary hosts within 60 days was taken as evidence for the presence of lymphoid precursor cells in the original inoculum. The experimental design may be summarized

as follows:




The fetal, newborn and adult tissues were obtained from A/HeJ mice. The primary hosts were adult male (BALB/c x A)$F_1$ mice and the secondary recipients were adult female (C57L x A)$F_1$ mice. All hosts were given 500 rad whole-body X radiation prior to the transfer of cells. Within each experimental group the mice were housed randomly, six to ten to a cage.

In addition, various tissues were taken from CBA/J embryos and injected into lethally X irradiated (840 rad) CBA-T6T6 mice which had been protected against the lethal effects of the radiation by two daily injections of urethan (0.2 ml of a 10% solution ip) (9). Approximately 30 days later these mice were killed and their tissues (thymus, spleen and bone marrow) were prepared for chromosome analysis. In most instances, at least 25 metaphase plates were scored for each tissue. The finding of donor cells in the thymus of the host was interpreted as indicating the presence of lymphoid stem cells in the inoculum (3, 5,10).

The pregnancies were surgically interrupted at various stages of gestation. The embryos and extra-embryonic tissues were dissected free of maternal tissue and blood. Identical organs or tissues from a single pregnancy (5-9 embryos) were pooled in cold TC 199 (Difco) and the cells were gently dissociated by mincing and aspiration through a 20-gauge needle. The age of the gestation was based on the following criteria: 7-8 days, a well developed ectoplacental cone and early membrane formation; 9 days, a well developed yolk sac, a conspicuous heart and no liver pigment; 10 days, early pigmentation in the liver; 11-12 days, a well pigmented liver and the absence of a differentiated thymus; 13-20 days, the relative size and development of the fetus. As there was at times considerable variation in the individual development of the embryos within a single pregnancy, the estimates in some instances may be in error by one half to one day. Except as noted below, one primary host was the recipient of the pooled identical organ from a single pregnancy. With gestations older than 16 days, the cell suspensions of placenta and liver were divided between two primary hosts.

Identical organs from newborn or older mice were pooled and the cells were gently dissociated as described above. Aliquots of the cell

suspensions were injected into sublethally irradiated hosts. With tissue donors one to seven days of age, there were from one to five donors for each recipient. At 14 days of age the ratio was approximately one to one. Outlined below is the cell dose (x $10^6$) given each recipient of tissues from older mice.

| AGE OF DONOR | LIVER | THYMUS | PEYER'S PATCH | LUNG | BONE MARROW | LN | SPL | BLOOD |
|---|---|---|---|---|---|---|---|---|
| 21 days | 2-30 | 15-20 | 1-2 | 3-7 | 4-10 | 0.6-6 | 1-23 | 7.5 |
| 42 or 84 days | 40-50 | 7-10 | 1-3 | 10-20 | 6-9 | 0.5-1 | 0.9- 1.6 | 0.25-1 |

In one series of experiments, tissues were removed from pregnant A/HeJ females (mated with A/HeJ males) during the 15th to 20th days of gestation and injected into primary hosts as described. In another experiment ovaries from A/HeJ mice were orthotopically transplanted to (BALB/c x A)$F_1$ females which had been castrated 10 days before. Three weeks later these females were mated with A/HeJ males. The few resulting pregnancies were interrupted between the 13th and 15th days of gestation and the embryos (8) and extra-embryonic tissues were injected into primary recipients as described above. In a final experiment, aliquots of a suspension of adult bone marrow cells were given to groups of thymectomized and normal recipients; sixty days later the lymphoid tissues of the primary hosts were injected into sublethally irradiated, nonoperated secondary recipients (4).

## RESULTS

Deaths were uncommon among the primary recipients of tissues from embryonic or newborn mice up to 14 days of age. Deaths did occur among the primary hosts given lymphoid tissues from older mice; in general, a given number of cells produced a mortality rate roughly proportional to the age of the donor (to 12 weeks of age). However, as relatively small numbers of mice were involved in each experiment, a precise age-dose-response relationship was not obtained. These results are not reported.

Deaths were rare among the secondary hosts given thymus cells from the primary recipients of embryonic tissues other than liver; therefore, these results are not reported. The mortality rates among the secondary recipients of tissues from newborn and adult mice were similar whether they had been given cells from the thymus or from the spleen and lymph nodes of the primary host. These data have been combined in Table II.

Uninjected controls, Table I, II: There were 25 deaths among the 656 irradiated mice which had received injections of lymphoid cells from the uninjected, irradiated primary controls. This represents a mortality rate of 3.8 ± 2.0% (range: 0-8%).

Seven to 9 day embryos, Table I: These early embryos were divided at the diaphragm, and each pole was assayed separately. There was only one death among the 32 secondary hosts. However, the preponderance of metaphase plates in the thymuses of the two irradiated hosts which had received the caudal half of 9 day embryos were of donor origin (the livers had been removed in one instance). No donor cells were found in the tissues of the irradiated host which had received the superior halves of the same embryos (Table III).

Yolk sac: There was one death among the 28 secondary recipients of 7 to 9 day yolk sac and 4 deaths among the 24 secondary hosts of 10 to 12 day membranes. The mortality rate sharply increased when yolk sac from 13 to 17 day embryos was used; there were no deaths among the secondary recipients of membranes from older fetuses.

In contrast to the results obtained above, donor cells were found in the thymuses of irradiated hosts in relatively large numbers when yolk sacs from 9 to 12 day gestations were used. The results obtained with membranes from older pregnancies closely paralleled those of the functional assay (Table III).

Three deaths occurred among the 4 secondary recipients of yolk sac from the eight 13 to 15 day A x A pregnancies which had been conceived in (BALB/c x A)$F_1$ females (liver, 2/3; placenta, 2/4; femur, 0/3; none, 0/12). The mortality rate among the secondary hosts of peripheral blood from pregnant A/HeJ females did not differ from that of the control group (Table II). These results tend to exclude contamination of the extra-fetal tissues by maternal blood as a significant factor in the results obtained with yolk sac and placenta.

Placenta: Deaths among the secondary recipients of placenta were first noted at 9 days and continued through the 16th day of pregnancy. The results obtained with the chromosome scoring were in close agreement with this.

Liver: The presence of lymphoid stem cells in the liver was first noted on the 10th day of gestation. The liver remained a major source of these cells until at least 21 days after birth. There was a good correlation between the 'G-V-H' results and the morphologic assay.

Thymus: The majority of deaths among the secondary hosts occurred

Table I. Sixty-day mortality(No./total) in sublethally x-irradiated secondary $F_1$ hybrids. The primary recipients received intraperitoneal injections of various embryonic tissues. The secondary hosts were given spleen cells from the primary hosts.

| Tissue Given Primary Host | Age of Embryo (Days) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 7-8 | 9 | 10 | 11-12 | 13-14 | 15-16 | 17-18 | 19-20 |
| Liver | | | 7/16* | 19/24 | 19/26 | 18/22 | 21/22 | 15/18 |
| Thymus | | | 0/2** | 6/16* ** | 6/22 | 3/18 | 0/10 | 0/14 |
| Gut | | | 0/4 | 0/12 | 2/17 | 0/14 | 3/24 | 2/32 |
| Lung | | | 0/4 | 0/16 | 0/18 | 11/22 | 15/18 | 14/26 |
| Femur | | | 0/2 | 0/6 | 0/22 | 0/14 | 2/16 | 5/21 |
| Spleen | | | | | | | 0/4 | 7/8 |
| Blood | | | | | | 0/4 | 1/6 | 1/14 |
| Skin | | | 0/6 | 0/10 | 0/16 | 1/14 | 0/10 | 0/10 |
| Yolk sac | 1/18 | 0/10 | 2/10* | 2/14 | 16/38 | 9/14 | 2/12 | 0/16 |
| Placenta | 0/12 | 2/10 | 8/14* | 14/22 | 28/44 | 10/16 | 0/24 | 1/52 |
| Above Diaphragm | 0/4 | 1/10 | | | | | | |
| Below Diaphragm | 0/12 | 0/6 | | | | | | |
| None | 1/20 | 0/34 | 2/51 | 2/66 | 3/56 | 2/45 | 1/40 | 1/48 |

*Some of these data have been reported previously(2). The addition of these data did not significantly alter the mortality rates.

**The upper trunk, excluding the lung and heart.

Table II. Sixty-day mortality (No./total) in sublethally x-irradiated secondary $F_1$ hybrids. The primary recipients received intraperitoneal injections of various newborn and adult tissues. The secondary hosts were given spleen and thymus cells from the primary hosts.

| Tissue Given Primary Host | Age of Donor (Days) 1-7 | 14 | 21 | 42 | 84 | Pregnant Female |
|---|---|---|---|---|---|---|
| Liver | 111/124 | 7/30 | 9/19 | 0/30 | 0/51 | |
| Thymus | 6/75 | 2/12 | 11/16 | 2/18 | 5/50 | 2/24 |
| Peyer's Patch | 21/69 | 0/9 | 0/22 | 5/30 | 2/27 | |
| Lung | 33/66 | 2/15 | 5/19 | 0/30 | 2/30 | |
| Skin | 0/27 | | | | | |
| Bone marrow | 49/57 | 29/30 | 9/14 | 20/30 | 34/80* 0/30* | |
| Lymph node | 8/30 | 0/9 | 5/10 | 1/6 | 0/16 | |
| Spleen | 74/94 | 10/21 | 6/16 | 0/27 | 0/24 | 0/12 |
| Blood | 13/36 | | 1/10 | 5/30 | 0/43 | 2/42 |
| None | 4/126 | | 0/19 | 0/30 | 7/85 | 2/36 |

*The primary hosts were thymectomized.

Table III. Percent donor cells in the THYMUS, spleen and bone marrow of lethally x-irradiated CBA-T6T6 mice 30 days after the intraperitoneal injection of various CBA embryonic tissues[1]

| Tissue injected | Age of Embryo (Days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 7 | 9 | 10 | 11-14 | 15 | 16-17 | 18-20 |
| Liver | | | 92, 100, 80 | 100, 88, 92 | 90, 100, 100 | 100, 84, 92 | 100, 100, 100 |
| | | | 0, 0, 0 | 90, 80, 96 | | 50, 76, 45 | 100, 100, 100 |
| | | | | 25.100. 84 | | | 100, 100, 100 |
| Thymus | | | | 0, 0, 0 | 0, 0, 0 | 12, 8, 0 | 4, 4, 0 |
| | | | | 4, 0, 0 | | | 0, 0, 0 |
| | | | | | | | 0, 0, 0 |
| Gut | | | 0, 28, 12 | 0, 0, 0 | 0, 0, 0 | 100,100,100 | 90, 70, 36 |
| | | | 0, 0, 0 | 0, 0, 0 | | | 90, 94, 28 |
| Lung | | | 90, 50, 4 | 0, 0, 0 | 32, 4, 0 | 80, 50, 40 | 4, 24, 0 |
| | | | | 0, 0, 0 | | | 8, 4, 0 |
| | | | | 0, 0, 0 | | | 80, 4, 0 |
| Femur | | | 0, 0, 0 | 0, 8, 0 | 62, 50, 4 | | 100, 96, 100 |
| | | | | 0 0 0 | | | 100, 84, 96 |
| Spleen | | | | | | | 0, 85, 25 |
| Blood | | | | | | | 100, 24, 16 |
| Skin | | | 0, 0, 0, | 0, 0, 0, | | 0, 0, 0 | 0, 0, 0 |
| | | | | 0, 0, 0 | | | 0, 0, 0 |
| Yolk sac | 0, 15, 0 | 0, 4, 0 | 84, 68, 16 | 33, 45, 24 | | 0, 30, 12 | 0, 8, 0 |
| | 0, 0, 0 | 90, 40, 0 | 80, 92, 16 | 100,100, 96 | | | 0, 0, 0 |
| | | | | 100,100,100 | | | 0, 33, 45 |
| Placenta | 0, 0, 4 | 90, 70, 4 | 90, 90, 100 | 0, 0, 0 | | 25, 50, 4 | 0, 60, 20 |
| | 0, 0, 0 | | 25, 75, 24 | 100, 60, 44 | | | 0, 0, 4 |
| | | | | 33, 28, 0 | | | 0, 8, 0 |
| | | | | 80, 33,100 | | | |
| Above diaphragm | 0, 0, 0 | 0, 0, 0 | 70, 100, 100 | 96, 92, 40 | | | |
| | | | 100, 90, 80 | | | | |
| | | | 0, 90, 100[a] | | | | |
| | | | 50, 80, 30 | | | | |
| Below diaphragm | 0, 0, 0 | 90, 80,100 | 100, 100, 100 | | | | |
| | | 100, 100,100[b] | 60, 60, 30[b] | | | | |

[1] The hosts were protected against the lethal effects of the radiation by two daily injections of urethan prior to irradiation.

[a] Without lung

[b] Without liver

when the tissue donors were between the 11th and 15th days of gestation. The mortality rate began to increase two days prior to the appearance of the thymus (the upper trunk was used). The mortality rate again increased sharply 21 days after birth. The morphologic studies did not correlate well with the results noted above. Donor cells were found rarely in the thymuses of the irradiated hosts, and their presence was unrelated to the age of the embryo.

Gut: There was little or no evidence for the presence in gut of lymphoid stem cells which have the potential to participate in cell-bound immune reactions until after birth; they were not found in the Peyer's patches after 7 days post-partum. The morphologic studies, however, consistently revealed donor cells in the host thymus when the gut was taken from embryos 16 days of age or older.

Lung: Deaths occurred among the secondary recipients of lung taken from embryos 15 days of age or older. The lung apparently remained a source of lymphoid stem cells until at least 21 days after birth. The findings above were supported by the morphologic studies. However, donor cells were also found in the host thymus when the cells were obtained from the lungs or upper trunks of 10 day embryos. They were not observed in the host thymus in the one instance where the lungs were removed from the upper trunks of the 10 day embryos or where the lungs were obtained from 11 to 14 day old embryos.

Femur and bone marrow: Deaths first occurred among the secondary hosts with the use of femurs from 18 to 20 day pregnancies. Donor cells were noted in the host thymus when cells from femurs 15 days or older were injected. The bone marrow remained a source of lymphoid stem cells until the mice were at least 12 weeks of age. Thymectomy of the primary hosts prevented deaths among the secondary recipients of adult bone marrow (0/30 vs 34/80)(4).

Spleen: The spleen was found to contain lymphoid stem cells during the last 2 days of gestation and until at least 21 days after birth. The one morphologic study performed did not reveal the presence of donor cells in the thymus of a mouse which had received cells from the spleens of 20 day fetuses.

Blood: Few deaths occurred among the secondary recipients of fetal blood cells. However, all of the metaphase plates in the thymus of an irradiated mouse given blood cells from 19 day embryos were of donor origin. Lymphoid stem cells were detected in the blood of 1 to 7 day old newborn mice but not thereafter.

Skin: There was no evidence by functional or morphologic criteria of the presence of lymphoid stem cells in the skin of embryonic or new born mice.

The above data has been graphically summarized in Figure 1. The curves were drawn to best fit the experimental data, but in the interest of clarity they were somewhat idealized.

## DISCUSSION

It would appear from these data that cells which have the potential to participate in 'delayed hypersensitivity' or cell-bound immune reactions may be present in the placenta as early as the 9th day of gestation; they are unquestionably there and in the liver on the following day. It is not clear from the results presented here whether these cells have their origin in the extra-fetal tissues and then migrate to the liver (or, perhaps, vice versa), or whether they arise independently in both sites. During the ensuing days of the pregnancy, lymphoid precursors are disseminated to the upper trunk, the thymus, yolk sac, lung, spleen and femur. Following birth, these stem cells are found in all of the lymphoid tissues of the newborn mouse. However, by six weeks after birth, the bone marrow appears to be the major or sole source of these cells.

A rather sharp increase in the mortality rate among the secondary recipients of newborn or adult thymus was observed when the tissue donors were 3 weeks of age. While these results may represent experimental variance, it was of interest to note that immunoglobulin production commences in the newborn mouse at about this time (11). This increase in the mortality rate then may be a reflection of an increased flow of lymphoid stem cells into the thymus in preparation for the onset of immunological maturity.

In general the morphologic assay used in these experiments for the detection of lymphoid stem cells correlated reasonably well with the results of the functional test. However, there were noteworthy exceptions. Embryonic gut gave little or no evidence of containing cells which have the potential to participate in cell-bound immune reactions; but, when dissociated gut cells from embryos 16 to 20 days of age were injected into irradiated mice, the majority of metaphase plates in the hosts' thymuses 30 days later were of donor origin. A similar situation was noted with 10 day lung, 20 day blood and yolk sac from 9 to 12 day embryos. These seemingly conflicting results may be attributable to (1) developmental differences between the strains of mice used, (2) differences in the sensitivities or specificities of the two test systems, (3) experimental error, or (4) the existence in the embryo of a line(s) of lymphoid stem cells which does not have the potential to participate in delayed hypersensitivity reactions but which is nonetheless thymus dependent. Recent work has shown that donor immunoglobulins ($\gamma$G2a and $\gamma$G1) are present in the sera of irradiated mice given gut cells

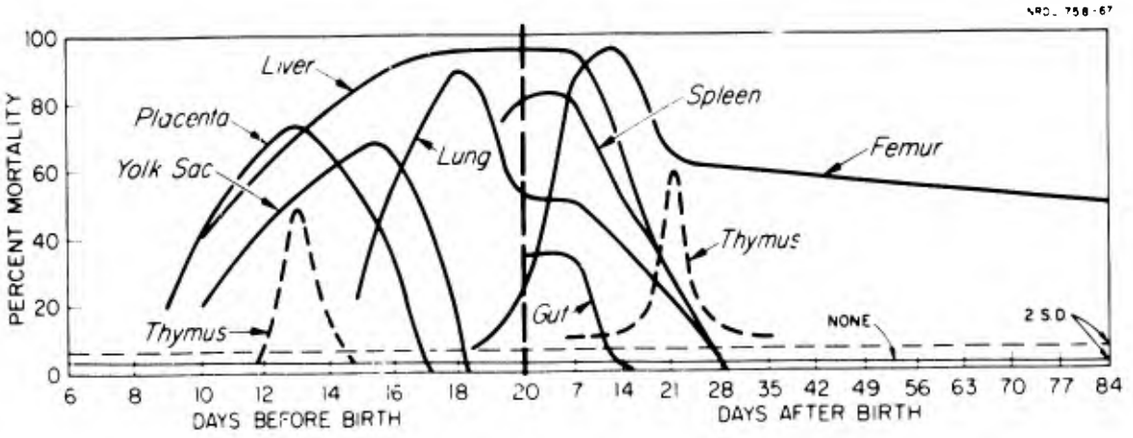



Figure 1. The relative graft-vs-host activity among secondary $F_1$ hybrid recipients of embryonic, newborn and adult tissues. The curves were drawn to best fit the experimental data, but in the interest of clarity they were somewhat idealized.

from 17 to 20 day embryos (12). Similarly cells from 10 and 11 day yolk sac have been shown to have the ability to produce immunoglobulins (13). The latter observations together with those presented above suggest that immunoglobulin producing cells (as opposed to those which mediate cell-bound immunity) may arise independently in the yolk sac on the 9th or 10th day of gestation, and that in the later stages of embryonic life these immunoglobulin producing cells may reside in the gut as a relatively pure cell population.

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13 ABSTRACT

The presence of potential immunologically competent cells in the various tissues of embryonic, newborn and adult mice was demonstrated by means of a modified 'graft-vs-host' method and by the injection of chromosomally marked cells into irradiated recipients. The results indicate that lymphoid stem cells which have the potential to participate in cell-bound immune reactions appear in the placenta and liver by the 9th or 10th day of gestation. Throughout pregnancy they are found in the liver. During the 11th to 14th days of gestation these stem cells are present in the upper trunk or in the thymus. On about the 15th day they appear in the lung and toward the end of the pregnancy in the fetal bone marrow and spleen. There was little or no evidence of the presence of lymphoid precursors capable of maturation to cells which mediate cell-bound immune responses in the gut prior to birth.

Following parturition lymphoid stem cells were present in the liver, Peyer's patches, lung, bone marrow, lymph nodes, spleen, blood and thymus. However, by six weeks after birth the bone marrow appeared to be the major or sole source of these cells.

Certain of the data suggest that immunoglobulin producing cells (as opposed to those which mediate cell-bound immunity) may arise in the yolk sac on the 9th or 10th day of gestation. Further, these immunoglobulin producing cells may reside in the gut as a relatively pure cell population after the 16th day of gestation.

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| Precursor<br>Immunocompetent cells<br>Graft-vs-host reactions<br>Embryogenesis | | | | | | |