# MATURATION OF HEMOLYSIN-PRODUCING CELL CLONES

## II. The Appearance and Localization of Precursor Units in Lymphoid Tissues of Neonatal Mice*

By GEORGE C. SAUNDERS, V.M.D., and DOUGLAS SWARTZENDRUBER

*(From the Department of Pathology, University of Colorado Medical Center, Denver, Colorado 80220)*

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Many mouse strains become immunocompetent to erythrocyte antigens during the 1st wk of life (1, 2). However, in vivo experimental techniques have, to date, been unable to answer the question as to where components of precursor units capable of interacting with erythrocyte antigens first arise. Using the modified Marbrook tissue culture technique (3, 4), we have undertaken a study of the kinetics of the appearance and maturation of precursor units in lymphoid tissues of neonatal mice. Tissues examined have been bone marrow, thymus, and spleen. These experiments have shown that cells capable of interacting with sheep erythrocyte antigens (SRBC) to produce hemolysin are usually first seen in the bone marrow and spleen, and, less frequently, in the thymus of mice 1–2 days old. However, in the reported system, these cells usually do not go on to produce a hemolysin-producing cell clone (HPCC). The first complete precursor units capable of interacting with erythrocyte antigen to produce a clone of hemolysin-producing cells are generally seen in the spleens of 3-day old mice. Much more rarely, such units are also found in the thymus of mice 2–3 days old. None of the cells isolated from the bone marrow, capable of interacting with erythrocyte antigen to produce hemolysin, have replicated to produce HPCC.

### *Materials and Methods*

*Mice.*—Colony bred, neonatal (12 hr to 7 day old), Swiss-Webster mice were used in all experiments. While the litter size did bear on the number of cells present per lymphoid organ at any given time (mice from smaller litters had larger lymphoid organs), the appearance of competence to SRBC was not obviously affected when the litter size ranged from 7–11 animals. Neonates from litter sizes of five or less do become competent about 24 hr earlier than do neonates from larger litters. The data to be reported here were derived from litters containing 7–11 neonates. Outbred mice were used in these experiments because (*a*) large litter sizes were consistently obtained, thus insuring enough lymphoid tissue for replicate cultures; (*b*) the length of the culture period (48 hr) is not sufficient to allow the weak graft-vs.-host reaction present in mice of this age to deleteriously affect the cell cultures.

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*Culture Reagents.*—Tissue culture reagents were as reported previously (4).

*Antigen.*—Sheep (CS1111) erythrocytes preserved in Alsever's solution obtained from the Colorado Serum Co., Denver, Colo., were washed three times in Hanks' balanced salt solution (HBSS). For in vivo experiments, neonatal mice were injected intraperitoneally with $1 \times 10^8$ washed SRBC in a volume of 0.05 ml. For in vitro experiments, a 5% suspension of washed SRBC was prepared by adding the appropriate volume of 1X stock medium (4) to the packed cells obtained after the final wash with HBSS.

*Lymphoid Tissues.*—Spleens and thymuses were aseptically removed in the usual manner. Bone marrow cells were aseptically obtained as follows: the femurs were amputated and the musculature removed. The epiphysis of each femur was cut and by using a pair of fine forceps, the marrow was stripped from the isolated femurs into cold HBSS. Cell suspensions were obtained from pooled lymphoid organs of each type by passing the cells through 100-mesh stainless steel screens into cold HBSS. Large clumps were allowed to settle out for 5 min, after which the remaining cells were placed into clean sterile tubes until used.

*Cell Culture.*—Cells were cultured, using the modified Marbrook technique, as previously reported (4). Variations were as follows: (*a*) 2X stock medium was added to equal volumes of 0.75% Agarose, instead of 0.70% Agarose. This slight increase in the Agarose concentration resulted in a firmer gel which facilitated such mechanical manipulations as pipetting and the washing of culture plates. (*b*) To increase the number of replicate cultures per litter and to keep relative cell density per culture constant, 35 mm × 10 mm plastic Petri dishes (Falcon No. 1008) were used, instead of 60 mm dishes. However, 60 mm Petri dishes were used for the kinetics experiment reported in Table IV.

The lower solid phase contained 1 ml of equal volumes of 2X stock medium and 0.75% Agarose solution. The upper solid phase contained 0.5 ml of equal volumes of 2X stock medium and 0.75% Agarose solution. In addition, the upper solid phase contained approximately $1 \times 10^8$ SRBC as the antigen. The liquid phase contained the appropriate number of lymphoid cells ($3.5–5 \times 10^6$) in 1 ml of 1X stock medium. Cultures were harvested after 48 hr of incubation at 37.5°C in a humidified atmosphere containing 7% $O_2$, 10% $CO_2$, and 83% nitrogen. Cultures were assayed for both the presence of precursor units and total plaque-forming cells per culture as previously described (4).

*Hemolysin Titers.*—Using Takatsy microtiter equipment, hemolysin titers were done on the sera obtained from immunized neonatal mice. 0.025 ml of serum was serially diluted in equal volumes of saline. 0.025 ml of guinea pig complement containing two 100% hemolytic units and 0.025 ml of 0.5% SRBC was added to each dilution. The tests were incubated at room temperature and were read 3 hr later. The reciprocal of the highest dilution causing complete lysis of the red cells was recorded as the titer.

## RESULTS

### *In Vivo Observations.*—

The ontogenesis of immunocompetence of neonatal mice to erythrocyte antigens has been described previously by others (1, 2). However, we wished to determine the onset of immunocompetence to SRBC antigens in mice bred under the environmental conditions of our animal colony. We also wished to compare the onset of in vivo immunocompetence with the in vitro results we obtained later. Litters of neonatal mice, 0–7 days old, were injected intraperitoneally with $1 \times 10^8$ SRBC. 4 days later, their spleens were removed and assayed for plaque-forming cells (PFC). Blood was also collected, and the serum was saved for hemolysin titers.

The results are shown in Table I. Mice injected at less than 3 days of age were unable to mount a detectable immune response to SRBC. However, mice

older than 3 days, when injected, did mount an immune response, as detected by both the presence of PFC and positive hemolysin titers. Reasons for the apparent low efficiency of the response observed in these animals will be discussed later.

*The Appearance of Background Cells.*—
Unimmunized mature mice usually have a number of cells present in their spleens which produce hemolysin (5–7). These cells have usually been referred to as background cells and may have arisen either in response to antigens which cross-react with erythrocytes or in response to Forssman antigen. It is also conceivable that background cells are antigen target cells. The presence of these cells has also led to speculation that so-called primary immune responses in vitro, described by Mishell and Dutton (8), Marbrook (3), and others (9, 10), have, in reality, been secondary responses. In order to have reasonable assurance that the observations to be presented herein are indeed related to primary exposure of lymphoid cells to SRBC antigens, a study has been done to see exactly when background cells first appear in mice bred and raised in our animal colony. Entire litters of mice were sacrificed at various times after birth and their background response to SRBC evaluated.

TABLE I

*Ontogeny of SRBC Immunocompetence in Vivo**

| Age at injection* | Nuc eated cells per spleen (×10⁶) (at sacrifice) | PFC per spleen | Hemolysin titer‡ |
|---|---|---|---|
| *days* | | | |
| 0 | 13.3 ± 2.1§ | 0.42 ± 0.38§ | 0 |
| 1 | 15.0 ± 1.9 | 0.93 ± 0.69 | 0 |
| 2 | 16.4 ± 0.8 | 1.81 ± 1.34 | 0 |
| 3 | 18.5 ± 2.5 | 74 ± 22.3 | 2 |
| 4 | 20.3 ± 3.6 | 402 ± 87.6 | 10 |
| 5 | 23.6 ± 2.7 | 913 ± 216 | 10 |
| 7 | 25.1 ± 3.2 | 1005 ± 342 | 40 |

* Swiss-Webster mice were injected intraperitoneally with 1 × $10^8$ SRBC and were sacrificed 4 days later. Each point represents the mean of at least 7 animals.

‡ Obtained from pooled sera of entire litter.

§ Standard error.

These results are summarized in Table II. Mice younger than 6 days of age had absolutely no detectable background cells in their spleens. One 7-day old mouse had one background cell in his spleen, while the rest of the litter was negative. Two-wk old mice had a few background cells in their spleens, but no detectable background cells in their bone marrow or thymuses.

*The Appearance of Immunocompetence in Vitro.*—
Bone marrow, spleens, and thymuses of neonatal mice were cultured in the presence of SRBC. After 48 hr of incubation, cultures were assayed for precursor units and total PFC. When the ratio of total PFC to precursor unit plaques was 1:1, we interpreted this to mean

that precursor cells maturated to the point of hemolysin production, but that replication of hemolysin-producing progeny failed to occur. When the ratio of total PFC was greater than 2:1, we assumed that replication had occurred. When replication did occur, the ratio of total PFC to precursor units was always at least 8:1.

The results of these experiments are summarized in Table III.

It can be seen that mice pass through a short period (about 24 hr) of total unresponsiveness to SRBC. However, 24–48 hr after birth, cells which maturate to the point of hemolysin production are seen in the bone marrow and spleen

TABLE II
*The Appearance of "Background" Cells in the Lymphoid Tissues of Neonatal Mice*

| Age | No. of mice tested | Nucleated cells per organ ($\times 10^6$) | Background cells per organ |
|---|---|---|---|
| *days* | | *Spleen* | |
| 3 | 9 | 13.1 ± 1.6* | 0 |
| 4 | 7 | 14.3 ± 1.9 | 0 |
| 5 | 8 | 15.5 ± 1.4 | 0 |
| 7 | 7 | 23.9 ± 2.1 | 0.14 ± 0.13* |
| 14 | 9 | 52.7 ± 5.3 | 3.78 ± 0.69 |
| | | *Bone marrow-femurs* | |
| 3 | 9 | 3.4 ± 0.8 | 0 |
| 14 | 9 | 10.3 ± 1.7 | 0 |
| | | *Thymus* | |
| 3 | 10 | — | 0 |
| 14 | 7 | — | 0 |

* Standard error.

In each case all cells from each organ ($10^7$ cells per plate) were incubated in the presence of SRBC for 2 hr, followed by the addition of complement for 60 min.

but they apparently do not undergo replication to produce hemolysin-forming progeny. Precursors of plaque-forming cells (PPFC) generally become capable of producing HPCC in mice 3–4 days old. It should be noted that HPCC were never obtained from bone marrow cultures and only very rarely from thymus cultures. However, 22 out of 56 thymus cultures from 3–7 day old mice contained PPFC which were at least capable of maturation.

Although the significance of these data will be more fully discussed later, it appears that the development of immunocompetence to SRBC in Swiss-Webster mice is at least a two-step process. First, cells appear that are capable of interacting with the antigen to maturate and produce antibody, but at this stage some factor (or factors) is missing which would enable these cells to replicate to form HPCC. The missing component (or components) rapidly

appears within 24–48 hr and PPFC are then able to replicate and to produce HPCC.

*Efficiency of Precursor Units in Neonatal Mice.—*

In order to compare the relative efficiency of precursor units contained in newly competent neonatal mouse spleens to the efficiency of precursor units contained in adult mouse spleens the following experiment was done. Spleens from both 5-day old and mature mice were cultured separately in the presence or absence of antigen. Cultures from both groups were harvested at 12 hr intervals for 48 hr and assayed for both precursor units and total PFC. The ratio between precursor units and total PFC in each group was compared at each time interval.

TABLE III

*In Vitro Ontogeny of Immunocompetence to SRBC by Swiss-Webster Mice*

| Age | No. of animals | No. of cultures | No. of cultures maturating* | No. of cultures replicating‡ |
|---|---|---|---|---|
| *days* | | | | |
| | | *Bone marrow* | | |
| 0 | 20 | 6 | 0 | 0 |
| 1 | 45 | 12 | 4 | 0 |
| 2 | 32 | 13 | 8 | 0 |
| 3 | 27 | 17 | 14 | 0 |
| 5 | 14 | 9 | 6 | 0 |
| 7 | 14 | 9 | 8 | 0 |
| | | *Thymus* | | |
| 0 | 21 | 21 | 0 | 0 |
| 1 | 47 | 31 | 1 | 0 |
| 2 | 45 | 48 | 5 | 1 |
| 3 | 38 | 22 | 9 | 1 |
| 5 | 9 | 9 | 5 | 1 |
| 7 | 22 | 25 | 8 | 0 |
| | | *Spleen* | | |
| 0 | 18 | 6 | 0 | 0 |
| 1 | 36 | 21 | 7 | 0 |
| 2 | 42 | 33 | 25 | 3 |
| 3 | 45 | 30 | 28 | 18 |
| 5 | 48 | 45 | 45 | 45 |
| 7 | 20 | 27 | 27 | 27 |

3–5 $\times$ $10^6$ cells per culture incubated in the presence of SRBC. Cultures assayed after 48 hr of incubation.

* Indicating at least the presence of PPFC.

‡ Indicating the presence of complete precursor units.

The results of this experiment are shown in Table IV. While the number of precursor units per culture was higher in the mature mice, the ratio of total PFC to precursor units was not significantly different between the two groups.

Unimmunized cultures contained very few PFC. This experiment implies that once functional precursor units appear, they respond to antigen in the same manner as those precursor units found in the mature animals. This data is in agreement with that of Silverstein (11).

## DISCUSSION

The low hemolysin titers, as well as the relatively low numbers of PFC obtained from neonatally injected mice, can be best explained by the low number of functional precursor units present, but probably not by the inefficiency of

TABLE IV

*Relative Efficiency* of Precursor Units in Neonatal Versus Mature Mice*

| Time | Precursor units per culture‡ | | PFC per culture‡ | | PFC per Precursor unit | | No. of doublings (approximate) | |
|---|---|---|---|---|---|---|---|---|
| | Neonatal | Mature | Neonatal | Mature | Neonatal | Mature | Neonatal | Mature |
| *hr* | | | | *With antigen* | | | | |
| 12 | 5.5 | 11 | 5.5 | 12.1 | 1.0 ± 0.09§ | 1.1 ± 0.18 | 0 | 0 |
| 24 | 5.5 | 10 | 20 | 41.0 | 3.64 ± 0.16 | 4.1 ± 0.34 | 2 | 2 |
| 36 | 5.5 | 12 | 50 | 106 | 9.1 ± 1.76 | 8.8 ± 1.53 | 3 | 3 |
| 48 | 5.0 | 11 | 78 | 181 | 15.6 ± 2.23 | 16.4 ± 1.94 | 4 | 4 |
| | | | | *Without antigen* | | | | |
| 12 | — | — | 0 | 1.25 | — | — | — | — |
| 24 | — | — | 0 | 3.75 | — | — | — | — |
| 36 | — | — | 0.5 | 8.0 | — | — | — | — |
| 48 | — | — | 1.25 | 14.75 | — | — | — | — |

* Based on the number of doublings per unit time under the same culture conditions.
‡ Mean of four cultures; $1.2 \times 10^7$ cells initially cultured.
§ Standard error.

these precursor units. Spleens of 5-day old Swiss-Webster mice usually contain about $1.5 \times 10^7$ nucleated cells and from 3 to 6 SRBC hemolysin precursor units per spleen, while adult Swiss-Webster mice have about $2 \times 10^8$ nucleated cells per spleen and from 200 to 400 precursor units capable of forming HPCC in response to SRBC (as determined by the Marbrook technique). Whether or not the avidity of the antibody produced by neonatal mice was equal to that of the adults was not tested.

Absence of any detectable background cells in the spleens of 0–6 day old mice provides some evidence that we are indeed measuring a primary immune response. Especially important in this regard is the fact that mice less than 24 hr old are totally unresponsive to SRBC in the system described. It would appear from this data that background cells may not be the antigen target cells,

since no detectable background cells were seen in neonatal mice (3–5 days old) which were competent to SRBC in vitro.

The correlation of in vivo data with the in vitro data is quite good. In both instances, the first generalized appearance of PFC resulting from proliferation of PPFC in response to SRBC is seen in mice first exposed to the antigen at the age of 3 days. By 5 days of age, the response is well developed in both systems.

Specificity controls were not done in mice less than 5 days old, mainly because of the lack of sufficient cells. However, the specificity of the Marbrook system has been documented previously (4). Negative controls were done when the spleens of 5-day old mice were cultured (Table IV).

We had hoped to find a better defined temporal sequence in regard to which lymphoid tissue contributes the first immunocompetent cells to SRBC antigen. While for all practical purposes, the first detectable complete precursor units (able to both maturate and replicate) are consistently seen in the spleen, the first cells able to interact with antigen to produce hemolysin appear simultaneously in the bone marrow and spleen. This observation may be explained by the fact that potentially immunocompetent stem cells from a common source may, in part, initially populate both organs. This source could be either the fetal yolk sac or fetal liver, both of which contain hemopoietic cells during fetal life. Another possibility is that the progenitor cells arise in the bone marrow and that some of these cells migrate to the spleen during late fetal life. Differentiation of the cells into immunocompetent cells can then occur independently in both organs.

The most interesting aspect of the data presented is the apparent stepwise development of competence to SRBC immunogens. Explanations of this phenomenon include the following. First, a two-step differentiation of progenitor cells may occur. In the first step, these cells differentiate into specific precursor cells capable of interacting with antigen to produce hemolysin. During the second stage of their maturation, the cell undergoes some transformation which enables it to replicate after antigen activation. This transformation may be under the influence of the thymus. An argument to support this view may be stated thus: while many neonatal bone marrow cultures contained cells which could produce hemolysin, none of these cells produced clones of hemolysin-forming progeny. In addition, in experiments not reported here, bone marrow cells from adult mice, cultured in the presence of SRBC also fail to produce HPCC. However, a few complete precursor units were found in the thymuses of neonatal animals. One could suggest, therefore, that PPFC which have arisen in the bone marrow, travel through the thymus where they gain the ability to replicate. Subsequently, these fully competent cells seed other lymphoid tissues. This argument would be better supported if complete precursor units were more frequently seen in the thymus.

A second, and in the light of the numerous recent reports (12–18) concerning

cell interaction during the immune response, perhaps more plausible explanation of stepwise appearance of competence is that functional precursor units contain two or more cellular components, at least one of which (PPFC) becomes functional before the other(s). One of us (4) originally believed that adherent cells may not be absolutely essential in initiating an in vitro antibody response in the Marbrook system. However, using the cell separation procedure described by Mosier (19), attempts to initiate an immune response in the Marbrook system using only nonadherent cells have failed, while combinations of the two cell populations (adherent and nonadherent) have been successful. However, 1:1 ratios of total PFC to plaques in the culture dish do occur when nonadherent cells are cultured, thus indicating that PPFC have maturated, but due to a lack of some property contained in the adherent cell population have failed to produce hemolysin-producing progeny. The enhancement of immunocompetence of neonatal mice to SRBC antigens by injecting them with large numbers of adult macrophages has also been reported (20).

Macrophages, therefore, are probably a component of the precursor unit. Presumably, their ability to function ontogenetically as such is delayed in comparison with the apparent earlier development of the PPFC. However, if macrophages are a component of the precursor unit, it would appear from the data presented here that they may not be required for antigen recognition by the PPFC, but that they may somehow interact with the PPFC and antigen to initiate replication of the PPFC, possibly by some type of membrane-associated feedback mechanism.

Why bone marrow, with a large adherent cell population, does not contain complete precursor units is puzzling. It is possible, however, that the adherent cells present in the bone marrow are functionally different from those contained in the spleen. Another possibility to explain incomplete competence of bone marrow would be that a third cell type is required (thymus derived?) for a functional precursor unit.

## SUMMARY

Cells capable of reacting with sheep erythrocyte (SRBC) antigen to maturate and produce hemolysin appear simultaneously in the bone marrow and spleen of 1-day old Swiss-Webster mice. However, hemolysin-producing cell clones (HPCC) do not result.

Complete functional precursor units generally appear in the spleens of mice older than 3 days. In vivo and in vitro data correlate well in this regard. Complete precursor units are not seen in the bone marrow and only very rarely in the thymus.

The efficiency of precursor units of neonatal mice when they become functional approximates that of the mature animal when based on the doubling time of plaque-forming cells (PFC).

Possible explanations of the initial appearance of incomplete precursor units have been discussed.

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