SPLENIC REPLENISHMENT OF SYNERGISTIC ABILITY TO
BONE MARROW AND THYMIC CELLS
OF NEONATALLY SPLENECTOMIZED CBA MICE*

BY R. A. BUCSI,* F. BOREK, AND J. R. BATTISTO
(From the Department of Microbiology and Immunology, Albert Einstein College of
Medicine, Bronx, New York 10461)
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Initiation of the humoral antibody response to certain antigens has been shown
to be dependent on the interaction of at least two types of lymphoid cells. Co-
operation between bone marrow (B) and thymic (T) cells was found to be essen-
tial for IgM and IgG responses to heterologous red blood cells and serum antigens,
respectively (1–4). Although a T cell factor has been shown to affect the ability
of B cells to interact (5, 6), all of the influences that allow development of the ca-
pacity of B and T cells to cooperate have yet to be elucidated. Indeed, several in-
stances are known where synergism normally seen between these two cells is absent.
For instance, the cooperative capacity has been found to be somewhat dependent on
age since T cells taken from animals less than 6 days of age have not yet acquired this
ability (7). Inducing tolerance during the neonatal stage also renders B and T cells
incapable of acting synergistically (4). In addition, in the humoral antibody response
of mice to Type III pneumococcal polysaccharide, B cells have been found to be inde-
pendent of T cell interaction (8, 9). Finally, we have found that with respect to IgM
synthesis towards sheep erythrocytes (SRBC), normal collaboration is absent in B
and T cells derived from hereditarily spleenless mice (10).

In the latter instance it was thought that possession of additional genetic
defects rather than absence of the spleen might account for inability of B and
T cells to act in synergy. This, however, was found not to be the case since co-
operative capacity of B and T cells of normal littermate B6.CBA mice spleen-
tomized neonatally was found to be absent (10). Thus, the spleen apparently
provides one of the essential influences on B and T cells that allows them to
cooperate to synthesize IgM. The experiments reported here were designed to
determine whether the results observed in hereditarily spleenless mice were ap-
pllicable in another strain of mouse where no immunogenetic defects have been

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‡ Present address: Department of Biology, University of Bridgeport, Bridgeport, Conn.
06607. Reprint requests may be sent to the Department of Microbiology and Immunology,
Albert Einstein College of Medicine, Bronx, N.Y. 10461.
1 Abbreviations used in this paper: B cells, bone marrow cells; BSS, balanced salt solution;
DPFC, direct plaque-forming cells; SRBC, sheep erythrocytes; T cells, thymic cells.
RESTORATION OF COOPERATIVE ABILITY TO B AND T CELLS

described. In the event that this were so, we wished to know if the defect in the cooperative capacity would be expressed equally by each population of cells as had been reported for the spleenless mouse. In addition, it was deemed essential to establish the critical interval after birth during which the spleen exerts its particular influence on B and T cells that causes them to become capable of collaboration. Finally, we wished to ascertain whether the defect in the cooperative capacity of these cells could be restored by in vivo replenishment of neonatally splenectomized mice with spleen cells.

Materials and Methods

Animals.—CBA/J mice obtained from Jackson Laboratory, Bar Harbor, Maine, were used throughout as donors and recipients of lymphoid cells. Neonatal splenectomies were performed at timed intervals after birth, after which the babies were placed with their mothers until weaning at 5-6 wk of age. Mice used as donors of cells were sacrificed at approximately 2-3 months postsplenectomy.

Spleen Replacement.—Spleen restoration was accomplished by two separate methods: (a) For some mice splenectomized at birth, each spleen that had been removed was separately minced in Hanks’ balanced salt solution (BSS), pulled through a syringe twice to dissociate cells, and each cell suspension was injected intraperitoneally into the original donor of the spleen. These mice were used as donors of B and T cells after a lapse of 2-3 months. (b) Other mice were splenectomized within 24 hr of birth and allowed to mature to 2 months of age before receiving from a littermate mouse the equivalent of one spleen dissociated in Hanks’ BSS. These replenished mice were sacrificed 1 month later when bone marrow and thymic cells were removed and transferred to X-irradiated mice.

Cell Transfers.—Mice intended as cell donors were sacrificed by cervical dislocation and their thymi and femurs were removed. Thymus cell suspensions were prepared by gently teasing the tissues in Earle’s BSS. Bone marrow cell suspensions were obtained by cutting the distal and proximal ends of each femur and flushing the interior with BSS. The cells were washed, adjusted to a concentration of 10⁷/0.1 ml, and injected intravenously via the retro-orbital sinuses of ether-anesthetized mice that had been subjected to 900 R whole body X-irradiation 2-4 hr before cell injections.

Immunizations.—Each cell recipient was immunized by a single intraperitoneal injection of 1 ml of 0.25% saline suspension of washed SRBC administered between 1 and 7 days after receipt of cells.

Hemolytic Plaque Assay.—Immunized mice were routinely sacrificed 4-5 days after the injection of SRBC. Their spleens were removed and cells suspended by gently pressing the tissue through a nylon, 60 mesh net into Hanks’ BSS. The cells were counted and examined for direct plaque-forming cells (DPFC) by the method of Jerne (11). Pooled guinea pig serum diluted 1:15 was the source of complement. Nonirradiated, immunized CBA mice possessing spleens served as positive controls.

RESULTS

To determine whether B and T cells from neonatally splenectomized CBA mice would be competent to act synergistically in vivo, they were removed when the animals had matured and transferred into syngeneic X-irradiated hosts. As controls B and T cells from normal littermate mice were transferred into separate animals. All were injected with SRBC intraperitoneally and their spleens assayed 4-5 days later for DPFC.
Synergism between B and T cells from normal CBA mice was readily demonstrated. The number of DPFC was found to be approximately five to seven times that achieved when B cells were transferred alone (Table I). In contrast, B and T cells taken from adult CBA mice that had been splenectomized within 24 hr of birth showed an inability to cooperate. This lack of synergism is readily apparent in the number of DPFC detected per million cells assayed. It is also seen when the total number of DPFC per spleen is compared with that value obtained using normal B and T cells.

To determine whether the depressed cooperation was due to a defect in one or both of the cells, normal B or T cells were combined with splenectomized T or B cells, respectively, and transferred into new recipients. When normal B cells were combined with splenectomized T cells, the same low number of DPFC per million was found as when both populations of cells were taken from splenectomized donors (Table I). In this instance, however, the number of DPFC per spleen was considerably smaller than that achieved when both B and T cells originated from splenectomized donors. When normal T cells were combined with neonatally splenectomized B cells, a slight degree of cooperation was seen in the number of DPFC per million. This was not evident, however, in the total number of DPFC per spleen. Thus, both B and T cells taken from neonatally splenectomized mice were found to be unable to cooperate, though the defect may be more pronounced in the T cell population.

To determine how soon after birth the spleen begins to exert its primary influence on B and T cell populations, splenectomy was performed at various neonatal intervals. B and T cells taken from adult mice splenectomized during the 2nd day after birth showed a similar inability to cooperate as cells taken from mice that had been splenectomized on the 1st day of life (Fig. 1). If, however, removal of the spleen was delayed for 6 days, a considerable improvement was

### TABLE I

| Description of adult donor of B cells | No. of syngeneic X-irradiated recipients | Mean DPFC (±SD) |
|-------------------------------------|----------------------------------------|-----------------|
| Normal                              | None given                             | 4               | 11.3 (5.0) | 650 (266) |
| Normal                              | Normal                                 | 3               | 50.0 (4.8) | 4552 (1603) |
| Neonatally splenectomized           | None given                             | 4               | 10.7 (1.5) | 227 (78) |
| Neonatally splenectomized           | Neonatally splenectomized              | 4               | 14.8 (4.0) | 1268 (624) |
| Normal                              | Neonatally splenectomized              | 5               | 13.6 (3.2) | 287 (135) |
| Neonatally splenectomized           | Normal                                 | 6               | 24.8 (11.2) | 377 (150) |
seen in the ability of B and T cells to cooperate in the synthesis of IgM. Thus, the ability of B and T cells to cooperate would appear to be strongly influenced by the presence of the spleen during the 1st wk of life.

Since removal of the spleen at an early age prevented the B and T cells from acting synergistically, restoration of this capacity was attempted by injecting dissociated spleens into neonatally splenectomized mice at various times. Restoration of cooperation was achieved when spleen suspensions were given immediately after splenectomy and the transfer of B and T cells deferred until 2 months later (Table II). In addition, the absent cooperative capacity could be replenished, even if the spleen administration was delayed for 2 months after neonatal splenectomy and the B and T cell transfer performed 1 month thereafter (Table II). Thus, although the spleen begins to influence B and T cells during the 1st wk of life, it apparently can be caused to do so at a later time, as well.

Fig. 1. Establishing the critical interval after birth for splenic influence on the ability of B and T cells to elicit the cooperative effect.
DISCUSSION

The observations presented here indicate that the spleen is a necessary factor for allowing bone marrow and thymic cells to develop the capacity to interact synergistically. Removing the spleens of neonatal CBA mice prevented their adult bone marrow and thymic cells from cooperating in the IgM response to sheep red blood cells. By combining the B or T cells from the neonatally splenectomized individuals with normal T or B cells, respectively, it was noted that a defect in cooperative ability was present in both cells derived from mice lacking spleens from birth, but that the deficiency may be greater in the T cell population. Previous work in this laboratory had shown that hereditarily spleenless mice had B and T cells which were equally defective in their cooperative ability (10). Since the neonatally splenectomized mice possessed spleens throughout fetal development and for a few hours before removal, some B cells may have been affected by the spleen's presence during this period and therefore became capable of interacting with T cells derived from normal mice. On the other hand, the T cell population derived from neonatally splenectomized mice apparently had not been so influenced by the presence of the spleen during these prenatal and very short, early postnatal stages. The defect in cooperative capacity seen in T cells from either the hereditarily spleenless or neonatally splenectomized mice appears to be comparable to the inactivity of T cells taken from mice less than 6 days of age as has been previously reported (7). In all three of these instances the absence of collaboration may be attributable to a lack of an initial stimulus emanating from the spleen during a critical period after birth. Indeed, we have found a 60% restoration in synergy can be achieved by delaying removal of the spleen until 6 days after birth. Thus, the T cell population appears to receive its initial impetus to cooperate from the spleen sometime during the 1st wk of life.

Restoration of the deleted cooperative capacity contingent upon neonatal
splenectomy could be accomplished by injecting the splenectomized animals with spleen cell suspensions. The replenishment was as effective when spleen cells were given 1 month after splenectomy as when they were given immediately after spleen removal at birth. Thus, the B and T cells would appear to be initially affected by the spleen during the early postnatal period. In addition, since their cooperative ability was found to be regenerated even several weeks after spleen removal, the splenic influence may be continuously expressed throughout the lifetime of an animal.

A possible analogy to this syngeneic spleen cell replacement in neonatally splenectomized mice is a condition that has been observed in human infants (12). Splenectomy in infants during the 1st yr of life resulted in serious infections except in cases of traumatic rupture of the spleen. The authors reported that in these latter instances the individuals did not experience recurrent infections. We view this difference as possibly being due to release of a number of spleen cells consequent to trauma that may be essential for maintaining the proper influence on B and T cells causing them to express full immunologic capacity.

The manner in which the spleen influences the capacity of B and T cells to act synergistically is not known. Indeed, in the synthesis of antibody the mechanism has not yet been elucidated by which these cells interact with one another as well as with macrophages that have processed antigen. Investigations have indicated that both the B and T cells possess antigen receptors (13-17) but that the T cell is not capable of producing antibody (18, 19). Dutton et al. (5) and Miller et al. (6) have found that T cells, when triggered by antigenic exposure, produce a humoral factor which stimulates bone marrow cells to synthesize antibody. The splenic influence described in our experiments must affect the B and T cells at a time before this particular antigenic encounter thus differentiating the splenic effect from the T cell factor. Further, our splenic effect must differ from the splenic macrophage supernatant factor described by Dutton et al. (20). This must be so since the recipient animals injected with B and T cells from either spleenless or normal animals, all have radiation-resistant macrophages capable of processing antigen. We are uncertain whether the splenic effect on B and T cells is attributable to a bone marrow colony promoting factor such as that reported by Metcalf et al. (21) or to a potentiating factor similar to that described by Janis and Bach (22).

One explanation that we are considering is that the spleen affects B and T cells in a manner that might cause them to “home” preferentially to the spleen. In the absence of such splenic influence fewer cells might find their way to spleens of X-irradiated recipients thereby disrupting the normal cooperative event. In a pilot experiment $^{51}$Cr-labeled B cells from normal and hereditarily spleenless mice were recovered from spleens of X-irradiated recipients in equal amounts; approximately 7% of each were present at 1 hr after transfer and about 8–10% at 24 hr. The data on the B cells from animals possessing spleens
are in agreement with those by Zatz and Lance (23). The fact that no difference in the number of B cells from both types of animals was observed does not rule out, however, the possibility that they were qualitatively different cells. In addition, we have not yet examined the homing behavior of T cells from both sorts of animals in the same way.

At present we are persuaded that a splenic imprint is not required for cooperation of cells in all immunological responses. This is apparent since hereditarily spleenless mice are fully capable of normal first- and second-set skin graft rejections (10), as well as the synthesis of certain G immunoglobulins (24). Investigations are in progress to determine the extent of the splenic influence on other immunological parameters as well as upon the mechanism of the splenic influence that allows B and T cells to interact.

SUMMARY

Bone marrow (B) and thymic (T) cells taken from adult mice that had been splenectomized within 24 hr of birth showed an inability to cooperate in the IgM response to sheep red blood cells. The defect in collaborative capacity was apparent in both sets of cells, but appeared to be more pronounced in the T cell population. Splenectomy performed at various neonatal intervals indicated that if removal of the spleen were delayed until 6 days after birth, B and T cells of the adult showed a 60% restoration in cooperation. Replenishment of the synergistic ability after neonatal splenectomy could be achieved by injecting spleen cells immediately after spleen removal or 2 months post-splenectomy.

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