IMMUNOLOGICAL ACTIVITY OF THYMUS AND THORACIC-DUCT LYMPHOCYTES

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The reduced immunological responsiveness of neonatally thymectomized rodents has been reversed far more effectively with cells from spleen, lymph nodes, and thoracic-duct lymph than with cells from either thymus or bone marrow.[1] In heavily irradiated mice, an inoculum containing a mixture of marrow and thymus cells allowed the production of more hemolysins against sheep erythrocytes than could be accounted for by summating the activities of each cell population alone.[2] Some type of interaction must thus take place between thymus cells, bone marrow cells, and antigen. In order to characterize the nature of this interaction one would first have to determine which cell type, thymus or bone marrow, was the *immediate* precursor of the antibody-forming cell.

In thymectomized irradiated mice protected with chromosomally marked bone marrow and grafted with chromosomally marked thymus tissue, both marrow-donor-type and thymus-donor-type cells were found dividing in the lymphoid tissues. The majority of dividing cells were of marrow donor origin,[3] but a sharp increase in the proportion of thymus-derived cells occurred for a short period of time after antigenic stimulation.[4] An attempt was made to determine the origin of the antibody-forming cell by using a transfer system in which both chromosome and isoantigenic markers were available. The results indicated that, although thymus-graft derived cells responded vigorously to antigenic stimulation by mitosis, the antibody-forming cells had the immunogenetic characteristics of the marrow donor.[4] However, it is known that the lymphocyte population of a thymus graft in a marrow-protected irradiated host is entirely replaced within two to three weeks by cells from the inoculated marrow.[3] Hence, although antibody-forming cells had the genotypic characteristics of the marrow donor, they could have been derived from cells that had first migrated through the thymus implant which, in these experiments, had been grafted 30 days prior to antigenic stimulation. Such an experimental design therefore failed to establish unequivocally which cell type was the immediate precursor of the antibody-forming cell: thymus or marrow cell.

In our laboratory we have attacked the problem by using cell suspensions rather than thymus grafts. A mixture of thymus and bone marrow cells from unrelated strains of mice was inoculated together with sheep erythrocytes into irradiated hosts. Under these conditions no hemolysin response was obtained, [5] suggesting that interaction does not take place between allogeneic cells immediately upon transfer to irradiated recipients. Neonatally thymectomized mice were then used as hosts of syngeneic or allogeneic thymus cells injected together with sheep erythrocytes. Since the marrow of neonatally thymectomized rodents

is normal according to several criteria,[6, 7] an interaction between thymus cells, sheep erythrocytes, and host cells (presumably marrow-derived) might be expected to occur and to be essentially similar in nature to that observed in heavily irradiated animals. In some experiments, thoracic-duct lymphocytes were used instead of thymus cells.

Materials and Methods.—Highly inbred CBA and C57BL mice were used throughout. Thymectomy or sham-operation was performed on mice less than 36 hours old as previously described.[8] Lymphocytes were obtained from the thoracic duct of 7- to 8-week-old mice by the method of Boak and Woodruff.[9] Cells from thymus, spleen, mesenteric lymph nodes, and bone marrow were suspended in tissue-culture medium 199 or Dulbecco's fluid. Cell-free thymus extracts were prepared according to the method of Metcalf.[10] A deep X-ray machine operating under conditions of 250 kv, 15 ma, 0.25 mm Cu, 1 mm Al filtration, and a focal skin distance of 30 cm with full backscatter conditions was used to irradiate mice which generally received 900 rads. Isoantisera were prepared by repeatedly injecting, at two-week intervals, adult CBA and C57BL mice with 10^7 cells from pooled thymus, spleen, and mesenteric lymph nodes of adult C57BL and CBA mice, respectively. The mice were bled one week after the last of four to six injections. The agar technique of Jerne[11] was used for detection of 19*S* hemolysin-forming cells.

Results.—Activity of thymus cells: The number of hemolysin-forming cells per spleen was 32,177 in sham-operated CBA mice and only 2356 in neonatally thymectomized CBA mice four to five days following an intravenous injection of 10^8 sheep erythrocytes at three to five weeks of age. Addition of syngeneic thymus cells to the inoculum of sheep erythrocytes elevated the peak response to 19,440 when 10 million cells were added and to 38,855 when 50 million cells were given (Fig. 1 and Table 1).

As seen in Table 1, C57BL thymus cells were also effective in elevating the peak hemolysin-forming cell response of neonatally thymectomized CBA mice when given together with sheep erythrocytes.

The reconstitutive capacity of large numbers of thymus cells was abolished by exposing the cells *in vitro* to 1000 rads of X irradiation (Table 1, group 5). Neonatally thymectomized mice injected intraperitoneally with 0.3 ml

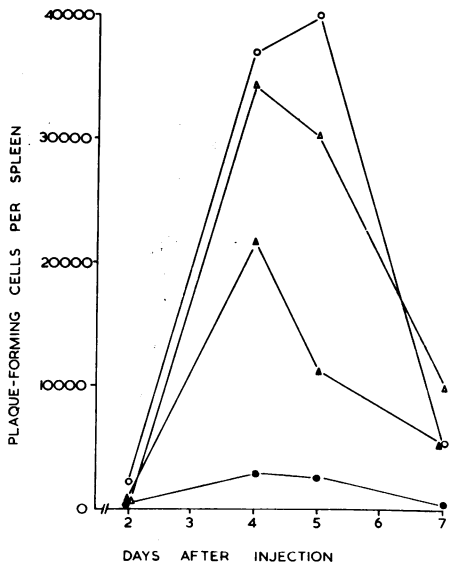

FIG. 1.—Number of hemolysin-forming cells produced per spleen in neonatally thymectomized CBA mice inoculated with CBA thymus cells and sheep erythrocytes. O, Thymectomized mice given 50×10^6 thymus cells; ▲, thymectomized mice given 10^7 thymus cells; ●, thymectomized mice not given thymus cells; △, sham-thymectomized mice not given thymus cells.

TABLE 1. *Hemolysin-forming cells in spleens of neonatally thymectomized mice inoculated with thymus cells and sheep erythrocytes 4 to 5 days before.*

Group	Recipient strain	Cells inoculated	No. of mice	Peak number of hemolysin-forming cells ± SE	*P* values (cf. group 1)*
1. Tx	CBA	SRBC only	16	2,356 ± 537	—
2. Tx	CBA	10^7 CBA T + SRBC	14	19,440 ± 4454	<0.05
3. Tx	CBA	5×10^7 CBA T + SRBC	22	38,855 ± 7448	<0.02
4. Tx	CBA	5×10^7 C57BL T + SRBC	8	18,106 ± 5307	<0.1
5. Tx	CBA	Irradiated CBA T + SRBC	4	1,160 ± 615	NS
6. Tx	CBA	CBA thymus extract + SRBC	5	1,802 ± 1155	NS
7. Tx	CBA	1.5×10^6 CBA TDL + SRBC	5	3,510 ± 1040	NS
8. Tx	CBA	10^7 CBA TDL + SRBC	9	20,244 ± 4165	<0.02
9. Tx	CBA	10^7 C57BL TDL + SRBC	10	30,120 ± 7791	<0.05
10. STx	CBA	SRBC only	36	32,177 ± 3550	<0.001

Abbreviations: Tx = thymectomized at birth; STx = sham-operated at birth; T = thymus cells; TDL = thoracic-duct lymphocytes; SRBC = 10^8 sheep erythrocytes; SE = standard error; NS = not significant.

* Calculated by student's *t* test.

saline cell-free thymus extracts (containing material from approximately one thymus lobe, or 100 million broken-up thymus cells) and at the same time intravenously with 10^8 sheep erythrocytes failed to produce more hemolysin-forming cells than neonatally thymectomized controls injected with sheep erythrocytes alone (Table 1, group 6). These experiments suggest that the activity of syngeneic thymus cells in this system is dependent not only on intact and undamaged cells but also on cells capable of dividing.

Activity of thoracic-duct cells: Thoracic-duct lymphocytes were inoculated intravenously with sheep erythrocytes into neonatally thymectomized mice. The peak response, measured by the number of hemolysin-forming cells per spleen, occurred four to five days later and is shown in Table 1. One and a half million thoracic-duct cells produced by no means as great a response as 10 million thymus lymphocytes in this system. This suggests that the effectiveness of thymus lymphocytes is unlikely to be attributable to contamination of the thymus cell suspension with lymphocytes of the type found recirculating from blood through lymph nodes to lymph and back to blood. Ten million thoracic duct cells from syngeneic donors elevated the peak response of thymectomized hosts to values comparable with those obtained when using 10 million thymus cells. A similar elevation was obtained with 10 million allogeneic thoracic-duct cells.

Identity of the precursor of the hemolysin-producing cell: The fact that allogeneic thymus or thoracic-duct cells were effective in elevating the number of antibody-forming cells produced in the spleen readily enables one to identify the immunogenetic character of the antibody-forming cells. We tested the capacity of specific isoantisera to eliminate hemolysin-forming cells from immunized C57BL and CBA mice and from neonatally thymectomized CBA or C57BL mice reconstituted with allogeneic thymus or thoracic duct cells. Aliquots of the respective spleen cell suspensions, taken at the height of the response, were incubated with complement and inactivated normal CBA and C57BL sera, and non-

TABLE 2. *Hemolysin-forming cells remaining after incubation of spleen cells with isoantisera.*

Source of spleen cells*	Number of hemolysin-forming cells obtained after incubation with: Normal mouse serum	Anti-C57BL serum	Anti-CBA serum
Normal CBA + SRBC	662	550 (−17%)†	68 (−90%)
Normal C57BL + SRBC	442	74 (−83%)	367 (−17%)
Tx CBA + 5 × 10^7 C57BL T + SRBC‡	460	407 (−12%)	37 (−92%)
Tx CBA + 10^7 C57BL TDL + SRBC‡	1612	1930 (0%)	60 (−96%)

* For abbreviations, see Table 1.
† Number in parentheses refers to per cent reduction.
‡ Only spleens pooled from high responders were tested.

specific and specific isoantisera. They were then washed and plated out to determine the number of hemolysin-forming cells remaining. The incubation of normal immunized mouse spleen cells with normal CBA or C57BL serum was associated with a loss of about 25 per cent of the number of antibody-forming cells. This probably resulted from nonspecific factors operating during the 30-minute incubation period and subsequent washing procedures. The experimental results were corrected for this "nonspecific loss" and are shown in Table 2. It will be seen that the specific isoantiserum reduced the number of hemolysin-forming cells in spleen from normal immunized mice by 83–90 per cent. In spleens from thymectomized CBA mice reconstituted with C57BL thymus cells, the number of hemolysin-forming cells was reduced by only 12 per cent with anti-C57BL serum but by as much as 92 per cent with anti-CBA serum. In spleens from thymectomized mice reconstituted with allogeneic thoracic-duct cells, the antiserum directed against the lymphocyte donor had no reducing capacity, whereas the antiserum directed against the thymectomized recipient reduced the number of antibody-forming cells by 96 per cent. It is clear, therefore, that the majority, if not all, of the antibody-forming cells produced in response to the inoculation of either thymus or thoracic-duct cells are derived not from the inoculated cells but from the host.

Interaction between thymus cells and antigen: The results so far indicate that thymus cells or thoracic-duct cells reconstitute immunological capacity in some way other than by providing the precursors of antibody-forming cells. The inactivity of irradiated thymus cells and cell-free thymus extracts implies that intact thymus cells and cell division may be essential. It has been previously reported[7] that a significant hemolysin-forming cell response occurred only in those irradiated mice receiving 10^7 syngeneic marrow cells, 10^8 sheep erythrocytes, and syngeneic spleen cells transferred from irradiated donors inoculated one week before with 10^8 syngeneic thymus cells and 10^8 sheep erythrocytes. No significant response occurred if bone marrow cells were not given to the second irradiated host nor if thymus cells had been incubated without sheep erythrocytes in the first irradiated host. This last finding suggested that thymus cells had to react with the antigen before interaction with bone marrow cells could produce a significant response. These results did not, however, establish whether the interaction between thymus cells and antigen was specific. Accordingly, groups of adult CBA mice were given 900 rads of total body irradiation and an intravenous injection of

TABLE 3. *Peak hemolysin-forming cell response in irradiated CBA mice inoculated with 10^7 CBA marrow cells, 10^8 sheep erythrocytes, and spleen cells transferred from irradiated CBA mice treated in various ways.*

Cells inoculated in first irradiated host	No. mice in group	No. hemolysin-forming cells in second irradiated host*
10^8 SRBC	5	310 ± 45†
10^8 CBA T	16	248 ± 32
10^8 CBA T + 10^8 RRBC	7	100 ± 16
10^8 CBA T + 10^8 HRBC	5	138 ± 37
10^9 CBA T + 10^8 SRBC	20	2103 ± 261

Abbreviations: T = thymus cells; SRBC = sheep erythrocytes; RRBC = rabbit erythrocytes; HRBC = horse erythrocytes.

* Hemolysin against sheep erythrocytes.

† Plus or minus standard error.

10^8 syngeneic thymus cells alone or together with 10^8 sheep erythrocytes, rabbit erythrocytes, or horse erythrocytes. These erythrocytes do not cross-react to any appreciable extent in immunological experiments.[12] One week later, spleen cells from these irradiated mice were inoculated together with 10^7 syngeneic marrow cells and 10^8 *sheep* erythrocytes into each mouse of a second set of irradiated CBA mice. The peak number of antisheep hemolysin-forming cells per spleen occurring in this group is shown in Table 3. A significant response occurred only in those irradiated mice that were recipients of spleen cells from irradiated donors incubating thymus cells and sheep erythrocytes. Incubation in the first host with noncross-reacting erythrocytes had the same effect as incubation without erythrocytes.

Discussion.—Syngeneic or allogeneic, thymus or thoracic-duct cells given to neonatally thymectomized mice, together with sheep erythrocytes, have bestowed upon their hosts the capacity to respond by producing as many or almost as many antibody-forming cells as can be expected in immunized sham-operated control mice of the same age. It might seem that the results obtained here with thymus cells contrast with those of previous studies which have indicated that the response of transplanted thymus cells to a primary antigenic challenge was weak and inconsistent. For instance, thymus lymphocytes injected with rat erythrocytes into irradiated mice produced much less antibody than a similar number of spleen cells.[13] Furthermore, in many experiments[1] it was found that intravenously injected thymus cells in doses of up to 20 million cells per mouse were much less effective immunologically than even 5 million spleen cells in thymectomized mice, although large numbers of thymus cells were effective.[14] It now appears that a number of reasons are available to account for the immunological inefficiency of thymus cell suspensions in some of these experiments. First, it must be remembered that there are immune responses which appear to be thymus-independent so that in these situations one would not expect thymus cells to produce a response. Secondly, in thymus-dependent systems, thymus cells given to irradiated hosts may not be effective owing to the inactivation, by irradiation, of an essential marrow cell-derived system. Spleen cells, on the other hand, would be effective because they are presumably a mixture of thymus-derived and bone marrow-derived cells. Thirdly, in many experiments, thymus

cells were given to neonatally thymectomized mice at or soon after birth and may not have been available in adequate numbers at the time the antigenic challenge was made some weeks later, possibly owing to their limited lifespan or to some previous immunological commitment. In the present experiments, thymus cells were given to neonatally thymectomized mice at the same time as the antigen.

The immediate precursor of the hemolysin-forming cell has been identified by immunogenetic methods as a cell type belonging to the host and not as a cell type derived by direct transformation of either thymus cells or thoracic-duct cells. It can be presumed that the host cell precursor is marrow-derived, since the bone marrow of neonatally thymectomized mice does not lack the precursors of hemolysin-forming cells.[7] It can further be surmised that in irradiated mice receiving mixtures of bone marrow, thymus, and sheep erythrocytes, the immediate precursors of the antibody-forming cells are marrow-derived. Syngeneic or allogeneic thoracic-duct small lymphocytes were found capable of restoring hemolysin production in *sublethally* irradiated rats,[15] but there has been no crucial demonstration that the donor lymphocytes developed into antibody-forming cells. The inoculated small lymphocytes, like the thymus or thoracic-duct cells in the present study, could have acted to enable *host* cells (that either escaped or recovered from the effects of irradiation) to differentiate to hemolysin-forming cells.

In the mouse, allogeneic thymus and thoracic-duct cells can produce a graft-versus-host reaction, thymus cells being less effective than thoracic-duct cells.[1] Because neonatally thymectomized CBA mice exhibit defects in homograft reactivity[1] and because there is a strong histocompatibility difference between C57BL and CBA, our reconstituted mice must have been experiencing some graft-versus-host activity. One could thus ask whether in the syngeneic combination some of the hemolysin-forming cells might not have been derived from donor lymphocytes. In such syngeneic systems immunogenetic methods cannot be used to determine the identity of the precursor of the antibody-forming cells. In preliminary experiments, two completely syngeneic systems were used: (1) CBA mice thymectomized at birth and inoculated with CBA/T6T6 thymus or thoracic-duct cells and sheep erythrocytes; (2) heavily irradiated CBA mice inoculated with CBA thymus cells, CBA/T6T6 marrow cells, and sheep cells. The cells of the CBA/T6T6 strain have a pair of readily identifiable unique chromosomes, which can be detected in individual antibody-forming cells by a recently developed cytological technique. All the dividing hemolysin-forming cells in the spleens of our mice examined individually so far have had the chromosome marker of the thymectomized host or of the marrow donor.[16]

Granted that the bone marrow provides the immediate precursor of the hemolysin-forming cell, a precursor that has *not* migrated through the thymus, what then is the role of the thymus or thoracic-duct cell in the conversion: precursor cell→antibody-forming cell; and to which cell lineage do "antigen-reactive cells" belong: thymus or bone marrow? It is unlikely that the effective thymus cell in the suspension used was a macrophage or an epithelial cell. Smears of such suspensions revealed few, if any, elements unequivocally identifiable as epithelial cells or macrophages. We shall therefore assume that the

effective cell type in the thymus is the lymphocyte. From the work of Gowans and his colleagues, we know that the effective cell type in thoracic-duct lymph is the small lymphocyte. Thus small lymphocytes from the thoracic duct and thymus may well belong to the same cell lineage: (1) both cell types reconstitute hemolysin-producing capacity, though not by transforming to antibody-forming cells as the present study indicates; (2) there is specifically a reduced number of recirculating small lymphocytes in neonatally thymectomized conventional[17] and germ-free mice;[18] (3) both thymus and thoracic-duct lymphocytes migrate to the periarteriolar lymphocyte sheaths of the spleen in irradiated mice,[15, 19, 20] and large pyroninophilic cells appear there in both cases in response to sheep erythrocytes;[15, 20] (4) thymus cell emigration studies have indicated that the number of cells leaving the thymus in young animals was sufficient to renew the entire circulating lymphocyte pool within a period of two–three months.[21]

A number of experimental findings indicate that there must be some specific interaction between thymus (or thoracic-duct) lymphocytes and antigens of the type exemplified by sheep erythrocytes in the mouse. In the experiments of Davies *et al.*,[4] thymus-graft-derived cells responded briskly to sheep erythrocytes by a burst of mitosis. In the irradiated hosts in the present study, the *specific* antigen had to be given to the thymus-incubating irradiated mice in order to obtain a significant response following transfer of spleen cells with added bone marrow and antigen to the second irradiated host. Incubation of thymus cells with noncross-reacting erythrocytes in the first host did not allow the second host to produce a significant response to sheep erythrocytes. Experiments with thoracic-duct lymphocytes from rats specifically tolerant of sheep erythrocytes have shown that the specific reactivity is a property of the lymphocytes.[15] All these findings suggest that thymus cells, or thoracic-duct cells, could react specifically to antigens, such as sheep erythrocytes in the mouse or rat, by differentiation to large pyroninophilic cells and mitosis, but not by giving rise to antibody-forming cells. The questions can now be asked: do "antigen-reactive cells" and antibody-forming cells belong to two quite distinct and separate cell lineages, the former thymus-derived and represented by the circulating pool small lymphocytes, the latter bone marrow-derived? If so, how do the two cell lineages interact in an immune response? Do thymus-derived cells react to antigen by producing an RNA-antigen complex necessary to trigger off the bone marrow-derived precursor of the antibody-forming cell? Or do they act as specific recognition agents capable of transferring, to essentially passive bone marrow-derived cells, the instructions required for the synthesis of specific antibody molecules? These questions can readily be answered in experiments employing allotype markers, the results of which will be reported later.

Summary.—Syngeneic or allogeneic, thymus or thoracic-duct cells were effective in substantially elevating the numbers of hemolysin-forming cells appearing in the spleens of neonatally thymectomized mice inoculated with sheep erythrocytes. The immediate precursor of the hemolysin-forming cell was identified, by means of immunogenetic techniques, as a cell type derived not from the inoculated thymus or thoracic-duct cells but from the thymectomized

host. By using a transfer system in heavily irradiated hosts in which thymus cells were temporally separated from bone marrow cells by a period of one week, it was shown that the thymus lymphocytes had first to react with the *specific* antigen before interaction with bone marrow cells could produce a significant hemolysin response. It is concluded (1) that the immediate precursor of the 19*S* hemolysin-forming cell is marrow-derived and (2) that thymus or thoracic-duct lymphocytes recognize antigen and interact with it in some way that triggers off differentiation of the essentially passive bone marrow-derived precursor cell to a specific antibody-forming cell.

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