STUDIES OF THYMOCYTOPOIESIS IN RATS AND MICE
I. Kinetics of Appearance of Thymocytes Using a Direct Intrathymic
Adoptive Transfer Assay for Thymocyte Precursors

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Several adoptive transfer systems for the detection of thymocyte progenitors
have been developed in rats and mice (1–6). Although these assays vary in
methodological detail, all use an i.v. route of cell transfer to study the ability of
hemopoietic precursors to repopulate the thymus of irradiated recipients. These
transfer systems have permitted significant progress in the identification, isolation,
and characterization of prothymocytes and in the general analysis of
thymocytopoiesis (e.g., 7–10). However, the i.v. transfer systems have several
properties that limit their usefulness for detailed studies of thymocyte develop-
ment. Among these limiting factors are low sensitivity, the requirement for cell
migration, and the engraftment of pluripotent hemopoietic stem cells and lymph-
oid stem cells in extrathymic sites. Consequently, in the i.v. systems, relatively
large numbers of precursor cells are required to generate detectable numbers of
donor-origin thymocytes; only those subsets of precursor cells that are able to
migrate from blood to thymus are detected; and the fine analysis of thymocyto-
poiesis is obscured by the continual seeding of donor-origin prothymocytes from
the chimeric bone marrow to the thymus of the adoptive host. These factors are
especially troublesome in the study of intrathymic precursor cells.

Herein, we describe a quantitative, intrathymic (i.t.)1 adoptive transfer assay
system that detects the generation of donor-origin thymocytes after the injection
of thymocyte precursors directly into the thymus of sublethally irradiated rats
and mice. The i.t. transfer assay, being independent of cell migration, is much
more sensitive than is the i.v. transfer assay, and can detect subsets of nonmigra-
tory as well as migratory thymocyte precursors. Furthermore, the i.t. transfer
assay is entirely selective for T-lineage precursor cells, and readily detects both
intrathymic and prethymic populations of precursor cells; yet neither hemo-

1 Abbreviations used in this paper: CFU-S, colony-forming unit–spleen (pluripotent hemopoietic
stem cell); i.t., intrathymic; TdT, terminal deoxynucleotidyl transferase.

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assay of prothymocytes by intrathymic injection. In Advances in Gene Technology. Molecular Biology
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prothymocytes in mice by intrathymic injection. Fed. Proc. 44:188a. (Abstr.)]
poietic nor lymphopoietic stem cell chimerism occurs at extrathymic sites. Because of these unique properties, we anticipate that the i.t. adoptive transfer system will greatly facilitate the identification and isolation of subsets of thymocyte precursors and the detailed analysis of thymocyte development and differentiation.

Materials and Methods

Animals. Rats of the BUF (RT-1^b, RT-7^b, IgK-1^a), M520 (RT-1^b, RT-7^b, IgK-1^a), LEW (RT-1^a, RT-7^b, IgK-1^a), and NBR (RT-1^b, RT-7^b, IgK-1^a) inbred strains and mice of C57BL/6 (H-2^b, Ly-1.2) inbred strain were obtained from the Animal Genetics and Production Branch, Division of Cancer Treatment, National Cancer Institute, Frederick, MD. Breeding pairs of the ALB (RT-1^b, RT-7^b, IgK-1^a) inbred rat strain and of the C57BL/6-Ly-1.1 (H-2^b, Ly-1.1) inbred mouse strain were obtained from the Small Animal Section, Division of Research Services, National Institutes of Health and from Dr. Edward Boyse, Memorial Sloan-Kettering Cancer Center, New York, respectively. Rats and mice used in these experiments were 5–7 wk old and were maintained on commercial chow and acidified, chlorinated water (pH 2.2; free chlorine, 5–10 ppm) ad libitum.

Antisera. Hybridoma cell lines secreting mAb against the RT-7.1 (BC84) and the presumptive RT-7.2 (8G6.1) rat pan-T cell alloantigens (11, 12) are maintained in our laboratory. The 8G6.1 hybridoma cell line was developed by and was the kind gift of Dr. D. M. Lubaroff, University of Iowa, Iowa City, IA. mAb against the IgK-1^a (MAR 80.2) and IgK-1^b (RG 11/15.1) kappa chain alloantigens, which are present on 95% of rat B cells (13–15), were a kind gift of Dr. G. A. Gutman (University of California, Irvine, CA). The mouse mAb OX8 and W3/25, which react with discrete T cell subsets in the rat (16), were obtained from Accurate Chemical & Scientific Corp., Westbury, NY. mAb against the Ly-1.1 and Ly-1.2 mouse pan-T cell alloantigens were obtained from New England Nuclear, Boston, MA. The hybridoma secreting the rat mAb against a common determinant of mouse Ly-2 (53-6.72) was obtained from the American Type Culture Collection (17). An affinity-purified IgG fraction of rabbit anti-terminal deoxynucleotidyl transferase (anti-TdT) (18, 19) was a generous gift of Dr. F. J. Bollum, Uniformed Services University of the Health Sciences, Bethesda, MD.

Cell Suspension Preparation. Bone marrow cells were prepared by flushing the marrow from the tibia and femur with cold media (RPMI 1640). Repeated gentle pipetting with a flame-polished pipette further dispersed the cells, which were then washed in cold medium. Thymus, spleen, and cervical lymph node cell suspensions were prepared by gently pressing the tissues through a stainless steel screen followed by washing in cold medium. Peripheral blood cells obtained from vessels in the tail (rat) or axilla (mouse) were collected in Alsevers solution. Spleen cell suspensions and peripheral blood cells were depleted of erythrocytes by hypotonic treatment with 0.168 M NH₄Cl. Cell viability was determined by exclusion of 0.1% trypan blue.

Immunofluorescence. Rat thymus cells were labeled in suspension with RT-7.1 or RT-7.2 antibodies and developed for indirect immunofluorescence with a fluorescein-conjugated F(ab')₂ fragment of a goat anti-rat IgG (H and L chain–specific) (Cappel Laboratories, Cochranville, PA) (1). Double immunofluorescence for surface RT-7 and intranuclear TdT was performed by preparing fixed smears of the RT-7-stained cells, developed with a rhodamine-conjugated F(ab')₂ fragment of goat anti-rat IgG (heavy and light chain specific), and by labeling with anti-TdT and fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories) (20).

Mouse thymus cells were labeled in suspension with Ly-1.1 or Ly-1.2 antisera and developed for indirect immunofluorescence with fluorescein-conjugated F(ab')₂ goat anti-mouse IgG (H and L chain–specific) (Cappel Laboratories). Double immunofluorescence for Ly-1.1 (donor-origin thymocytes) and TdT was performed as above using a rhodamine-conjugated F(ab')₂ fragment of goat anti-mouse IgG to develop the anti-Ly-1.1 antibodies and fluorescein-conjugated goat anti-rabbit IgG to develop the anti-TdT antibodies (21).

Donor- and host-origin rat, splenic, kappa chain–bearing B cells were quantified using
the mAb MAR 80.2 (anti-IgK-1\(^+\)) and RG 11/15.1 (anti-IgK-1\(^-\)). Labeled cells were developed for fluorescence analysis using a fluorescein-conjugated F(ab')\(_2\) goat anti-mouse IgG (H and L chain-specific) that had been passed over a normal rat Ig Sepharose 4B affinity column to remove crossreacting antibodies (1).

Double immunofluorescence for the rat RT-7.1 and OX8 or W3/25 antigens was performed as follows: spleen or lymph node cells were first labeled with fluorescein-conjugated anti-RT-7.1 to identify donor-origin cells. Cells were then labeled with OX8 or W3/25 mAb and developed for indirect immunofluorescence using rhodamine-conjugated F(ab')\(_2\) goat anti-mouse IgG (H and L chain-specific) that had been passed over a normal rat Ig Sepharose 4B affinity column to remove crossreacting antibodies (1).

Double immunofluorescence for the mouse Ly-1.1 and Ly-2 antigens was performed as follows: spleen cells were first labeled with mouse anti-Ly-1.1 and developed for indirect immunofluorescence with fluorescein-conjugated goat anti-mouse IgG that had been passed over a normal rat Ig Sepharose 4B affinity column to remove crossreacting antibodies. Cells were then labeled with rat anti-Ly-2 and developed for indirect immunofluorescence using rhodamine-conjugated F(ab')\(_2\) goat anti-rat IgG that had been passed over a normal mouse IgG Sepharose 4B affinity column to remove crossreacting antibodies (1).

Cell suspensions were analyzed according to low angle light scatter and relative fluorescence intensity on a FACS (FACS IV; Becton Dickinson Immunocytometry Systems, Sunnyvale, CA) equipped with a dual-fluorescence compensation network (1, 9). Cell smears were analyzed visually using a Zeiss Universal fluorescence microscope equipped with narrow band filters for rhodamine and fluorescein.

**Irradiation.** Recipients received total body irradiation from a \(^{137}\)Cs source (110 rad/min) (Gamma Cell 40 Irradiator; Atomic Energy of Canada, Ltd., Ottawa, Canada) 2–6 h before receiving bone marrow cells.

**In Vivo CFU-S.** In vivo spleen CFU (CFU-S) activity was determined by counting spleen colonies 12 d (rats) or 9 d (mice) after the intravenous or intrathymic transfer of bone marrow cells into irradiated (700–750 rad), histocompatible, 5–6-wk-old recipients (22, 23).

**Intrathymic Adoptive Transfer Assay for Prothymocytes.** Rats and mice were anesthetized with a mixture of ketamine hydrochloride (1.20 mg/100 g body weight) (Ketaset; Bristol Laboratories Div., Bristol-Myers Co., Syracuse, NY) and acepromazine maleate (0.12 mg/100 g body weight) (PromAce; Fort Dodge Laboratories, Fort Dodge, IA), a muscle relaxant, injected i.p. A midline incision was made in the skin overlying the lower cervical and upper thoracic region, and the upper third of the sternum was bisected longitudinally with fine scissors to expose the thymus. Suspensions of bone marrow, thymus, spleen, lymph node, or peripheral blood cells were injected at two sites in the anterior superior portion of each thymus lobe (10 \(\mu\)l/site) using a 1-ml syringe equipped with a 28-gauge needle and mounted on a Tridak Stepper (Indicon Inc., Brookfield Center, CT). The depth of the injection was not critical as long as the tip of the needle did not pass through the thymus and the injected material did not escape through the needle track. The incision was closed with wound clips. When set up on an assembly line basis with two operators, ~24 animals can be injected per hour.

At various times after reconstitution, the recipients were killed and the percentages of donor- and host-origin cells were quantified by immunofluorescence analysis on the FACS. Donor-recipient strain combinations are reported in the text. Except when indicated, the results of individual experiments are presented. However, all experiments were repeated two to six times, and comparable results were obtained.

**Intravenous Adoptive Transfer Assay for Prothymocytes.** Details of this assay in rats and mice have been described previously (1–6). Briefly, irradiated recipients were injected intravenously via the tail vein with histocompatible, RT-7-disparate (rats) or Ly-1-disparate (mice) donor bone marrow cells. Donor-recipient strain combinations for individual experiments are reported in the text. The recipients were killed and percentages of donor- and host-origin RT-7\(^+\) (rats) or Ly-1\(^+\) (mice) thymocytes and T cells were quantified by immunofluorescence analysis on the FACS at various times after reconstitution.
Results

A detailed description of the i.t. adoptive transfer system in rats is presented below. Similar results were observed in mice so only a brief description of these experiments is provided, with complete data given in the figures and tables.

*Time Dependence of Thymocyte Regeneration.* The appearance of donor-origin thymocytes in sublethally irradiated (600 rad) recipients as a function of time was determined after the i.t. or i.v. injection of bone marrow cells. In the rat, significant numbers of donor-origin thymocytes were consistently detected by day 12 after the i.t. transfer of $10^6$ bone marrow cells, and by day 14 after the i.v. transfer of $2.5 \times 10^7$ bone marrow cells (Fig. 1A). Thereafter, a parallel
linear increase in the absolute number of donor-origin thymocytes occurred until
days 20 or 22, respectively. $\sim 8 \times 10^6$ donor-origin thymocytes were present on
day 12 and $1.25 \times 10^6$ on day 20 in i.t.-injected recipients, representing 1.6 and
19.9%, respectively, of total thymocytes. However, between days 20 and 41,
donor-origin thymocytes in i.t.-injected recipients decreased to undetectable
levels ($<1\%; <5 \times 10^6$), where they remained for at least 3 mo. In contrast, the
i.v. injection of bone marrow cells resulted in the establishment of a permanent
chimera.

In the mouse, significant numbers of donor-origin thymocytes appeared on
day 14 after the i.t. transfer of $10^5$ and the i.v. transfer of $2.5 \times 10^6$ bone marrow
cells. From day 14 through day 26 an exponential and superimposable increase
in the number of donor-origin thymocytes occurred in both assay systems (Fig.
$1B$). This was followed in the i.t. system by a decrease in the donor-origin
thymocyte number from $6.37 \times 10^7$ on day 26 to $5.7 \times 10^6$ on day 35; whereas,
in the i.v. system, donor-origin thymocytes reached a plateau by day 26 and
formed a permanent chimera.

In both the rat and mouse systems, examination of cell suspensions showed
that $\sim 70\%$ of the donor-origin thymocytes were TdT$^+$ after either i.t. or i.v.
injection of bone marrow cells, which approximates normal values (18).

Dose Dependence of Thymocyte Regeneration. In the rat, there was a direct
relationship between the number of bone marrow cells injected i.t. and the
absolute number of donor-origin thymocytes observed in irradiated recipients
between day 14 and day 20, i.e., during the linear phase of the time response
(see Fig. 1 A). Fig. 2A shows the results for day 14. The dose response was linear
in the range of $0.1 \times 10^6$--$1.5 \times 10^6$ bone marrow cells. No further increase in
the number of donor-origin thymocytes was observed at higher doses of bone
marrow cells.

Since the slopes of the time-response curves after the i.t. and i.v. transfer of
bone marrow cells are parallel in the rat (Fig. 1 A), the relative sensitivities of the
two assays can be compared directly. Thus, in a representative experiment shown
in Fig. 2A, as few as $5 \times 10^4$ bone marrow cells injected i.t. were able to generate
the same number of donor-origin thymocytes (1.9 $\times 10^7$) by day 14, as were $2.5
\times 10^7$ bone marrow cells injected i.v. The results of several similar experiments
showed that the i.t. assay is $\sim 40$-fold more sensitive (range, 26--55-fold) than is
the i.v. assay in detecting hemopoietic thymocyte progenitors in the rat when
compared on the same day (Table I). When the relative sensitivity was calculated
allowing for the 2 d-greater lag for the appearance of donor-origin thymocytes
in the i.v. assay (Fig. 1 A), the i.t. assay was $\sim 18$-fold more sensitive (range, 12.5--
27.8) than was the i.v. assay.

In the mouse, the dose response of the i.t. assay was $\sim 10$-fold lower than that
in the rat, being linear in the range of $0.75 \times 10^5$--$2.50 \times 10^5$ bone marrow cells
on day 17 (Fig. 2B). In addition, the i.t. assay is $\sim 25$-fold more sensitive than is
the i.v. assay when tested on day 17 (Table I).

Irradiation Dependence of Thymocyte Regeneration. The results in Table II show
that the threshold dose of irradiation for the generation of donor-origin thy-
mocytes is $\sim 400$ rad in the mouse and 500 rad in the rat for both the i.t. and
i.v. assays. Doses of $\geq 700$ rad resulted in the sickness or death of the i.t.- but not
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FIGURE 2. The kinetics of appearance of donor-origin thymocytes as a function of dose of bone marrow cells injected i.t. into irradiated recipients. (A) Irradiated (600 rad) BUF rats were injected i.t. with M520 bone marrow cells. The dose-response relationship was linear (linear regression coefficient $r^2 = 0.97$; slope $= 31.88$) in the dose range of $0.1 \times 10^6 - 1.5 \times 10^6$ bone marrow cells when examined during the linear phase of the time response (day 14). Recipients injected with $2.5 \times 10^6$ or $5.0 \times 10^6$ bone marrow cells i.v. yielded $1.9 \times 10^7$ and $4.0 \times 10^7$ donor-origin thymocytes, respectively, demonstrating that, at these doses and time point, the i.t. transfer system is 41-49-fold more sensitive than is the i.v. adoptive transfer system (see Table I). (B) Irradiated (600 rad) C57BL/6 mice were injected i.t. with C57BL/6-Ly-1.1 bone marrow cells. The dose-response relationship was linear ($r^2 = 0.95$; slope $= 135.98$) in the dose range of $0.75 \times 10^5 - 2.5 \times 10^6$ bone marrow cells (closed circles, day 17; open circles, day 14). Recipients injected with $2.5 \times 10^6$ bone marrow cells i.v. yielded $6.8 \times 10^6$ donor-origin thymocytes, demonstrating that, at this dose and time point the i.t. transfer system is -25-fold more sensitive than is the i.v. transfer system (see Table I).

the i.v.-injected animals. Therefore, as indicated below, when irradiation doses >600 rad were used, limiting numbers of syngeneic host-origin bone marrow cells were given i.v. to rescue the i.t.-injected animals, since it appears that there is no significant seeding of donor-origin bone marrow cells to the recipient's bone marrow in the i.t. system.

Generation of Peripheral T Cells. In the rat system, $10^7$ bone marrow cells were transferred either i.t. or i.v. into lethally (750 rad) irradiated recipients. This high irradiation dose was used to reduce the number of surviving host-
Relative Sensitivity of Intrathymic and Intravenous Assay Systems for Detecting Thymocyte Progenitors in Bone Marrow

| Animal model | Days after bone marrow cell transfer | Ratio of intrathymic to intravenous assays |
|--------------|--------------------------------------|------------------------------------------|
| Rat          | 14                                   | 42                                       |
|              | 14                                   | 31                                       |
|              | 14                                   | 48                                       |
|              | 14                                   | 50                                       |
|              | 18                                   | 26                                       |
|              | 19                                   | 55                                       |
|              |                                      | **42.0 ± 11.4**                           |
| Mouse        | 17                                   | 24                                       |

Relative sensitivities of assays were determined by comparing the number of bone marrow cells required to produce equivalent numbers of donor-origin thymocytes on a given day after injection i.t. or i.v. (see Fig. 2, A and B). To eliminate interexperimental variation, a dose-response curve for the i.t. assay was constructed for each experiment.

* Mean ± SD. If one assumes a 48-h increased lag period for the i.v. assay in the rat (see Fig. 1A), the relative sensitivity of the i.t. assay is 18.5 (±5.4) times greater than that of the i.v. assay.

Effect of Irradiation Dose on Generation of Donor-origin Thymocytes in Intrathymic Adoptive Transfer System

| Dose of irradiation | Route of bone marrow cell transfer | Number of donor-origin thymocytes per thymus (×10^-6) |
|---------------------|------------------------------------|------------------------------------------------------|
|                     |                                    | Rat*                                                 |
| rad                 |                                    | Mouse*                                               |
| 0                   | i.v.                               | <1                                                   |
|                     | i.t.                               | <1                                                   |
| 500                 | i.v.                               | <1                                                   |
|                     | i.t.                               | 4.5 ± 3.7                                            |
| 400                 | i.v.                               | <1                                                   |
|                     | i.t.                               | 6.9 ± 8.8                                            |
| 500                 | i.v.                               | <1                                                   |
|                     | i.t.                               | 7.5 ± 3.9                                            |
| 600                 | i.v.                               | 8.3 ± 1.9                                            |
|                     | i.t.                               | 47.6 ± 26.3                                          |
|                     |                                    | 12.5 ± 8.4                                           |
|                     |                                    | 17.6 ± 15.3                                          |
|                     |                                    | 3.7 ± 1.1                                            |
|                     |                                    | 44.8 ± 14.7                                          |
|                     |                                    | 28.6 ± 3.0                                           |

* BUF (RT-7b) recipients were irradiated and injected within 2–6 h with 10 × 10^6 M520 (RT-7b) bone marrow cells i.t. or i.v. Donor-origin thymocytes (RT-7.1) were quantified by immunofluorescence analysis on the FACS 16 d later. Results represent the mean ± SD of three animals per group. Values of <1 indicate that no donor-origin thymocytes were detected.

‡ C57BL/6 recipients (Ly-1.2) were irradiated and injected within 2–6 h with C57BL/6-Ly-1.1 bone marrow cells i.t. (0.1 × 10^6) or i.v. (2.5 × 10^6). Donor-origin thymocytes (Ly-1.1) were quantified by immunofluorescence analysis on the FACS 17 d later.
origin T cells, thereby facilitating the detection of donor-origin T cells. Each recipient also received a hematopoietic rescue dose of $5 \times 10^6$ syngeneic bone marrow cells.

By day 30, significant numbers of donor-origin T cells were detected in the spleen of both the i.t.- and i.v.-injected recipients (Table III). Donor-origin T cells were also detected in the lymph nodes of these animals (data not shown). The antigenic phenotypes of the donor-origin T cells were determined by double immunofluorescence using mAb W3/25 and OX8 as selective T cell subset markers, and mAb against RT-7.1 as a marker for donor-origin T cells. The ratio of presumptive helper/inducer (W3/25+) to cytotoxic/suppressor (OX8+) donor-origin T cell subsets were comparable in the i.t.- and i.v.-injected recipients, and approximated the expected ratio of 1:1 in normal nonirradiated controls (Table III). Comparable results were observed in the mouse system, in which the i.t. transfer of $10^5$ bone marrow cells resulted in the generation of detectable numbers of donor-origin T cells by about day 30 (Table III).

**Absence of Donor Origin Hemopoietic Spleen Colonies or B Cell Regeneration in i.t.-injected Recipients.** No donor-origin hemopoietic colonies were observed in the spleens of irradiated (750 rad) recipients that were injected i.t. with $10^6$ or $10^7$ rat bone marrow cells or $0.75 \times 10^6$-$2.50 \times 10^6$ mouse bone marrow cells (Table IV). In contrast, the spleens of rats and mice injected i.v. with $10^6$ and $10^5$ bone marrow cells, respectively, contained the expected numbers of colonies.

Similarly, as determined by mAb against rat kappa chain Ig allotypes (2, 13-

| Animal model | Route of bone marrow cell transfer* | Number of cells transferred ($\times 10^6$) | Percentage of donor-origin T cells per spleen | Number of donor-origin T cells per spleen ($\times 10^6$) | Ratio of donor-origin T helper to T suppressor cells in spleen |
|--------------|------------------------------------|------------------------------------------|-------------------------------------------|------------------------------------------------|--------------------------------------------------|
| Rat          | i.v.                               | 10                                       | 9.5 ± 0.9                                 | 13.8 ± 8.1                                    | 1:1                                              |
|              | i.t.                               | 10                                       | 8.5 ± 0.6                                 | 7.6 ± 2.3                                     | 0.7:1                                           |
|              | Uninjected controls                | --                                       | --                                        | --                                            | 0.9:1                                           |
| Mouse        | i.v.                               | 2.5                                      | 7.0                                       | 2.8                                           | 2.4:1                                           |
|              | i.t.                               | 0.1                                      | 3.1                                       | 0.9                                           | 1:1                                              |
|              | Uninjected controls                | --                                       | --                                        | --                                            | 2:1                                              |

* Irradiated (750 rad) BUF (RT-77) rats were injected i.t. or i.v. with M520 (RT-77) bone marrow cells. Both groups also received a hematopoietic rescue dose of $5 \times 10^6$ syngeneic (BUF) bone marrow cells i.v. Donor-origin T cells (RT-7.1) in the spleen were quantified by immunofluorescence analysis on the FACS 30 d after reconstitution. Irradiated (700 rad) C57BL/6 (Ly-1.2) mice were injected i.t. or i.v. with C57BL/6-Ly 1.1 bone marrow cells. Both groups also received a hematopoietic rescue dose of $0.5 \times 10^6$ syngeneic C57BL/6 (Ly-1.2) bone marrow cells i.v. Donor-origin T cells (Ly-1.1) in the spleen were quantified 29 d after reconstitution. Control animals were age- and sex-matched, unirradiated M520 rats and C57BL/6-Ly 1.1 mice, respectively.

1 Determined by double immunofluorescence, for RT-7.1 (donor-origin) and W3/25 (helper/inducer) or OX8 (suppressor/cytotoxic) T cell subsets in the rat, and for Ly-1.1 (donor-origin T cells) and Ly-2+ (helper/inducer) and Ly-2+ (suppressor/cytotoxic) T cell subsets in the mouse.
TABLE IV
Absence of Donor-origin Hemopoietic Spleen Colonies and B Lymphocyte Regeneration in Intrathymic Adoptive Transfer System

| Animal model | Days after bone marrow cell transfer | Route of bone marrow cell transfer | Number of bone marrow cells transferred (×10⁶) | Number of donor-origin thymocytes per thymus (×10⁶) | Number of hemopoietic spleen colonies | Number of donor-origin B lymphocytes per spleen (×10⁶) |
|--------------|-------------------------------------|-----------------------------------|-----------------------------------------------|--------------------------------------------------|-------------------------------------|--------------------------------------------------|
| Rat          | 12                                  | i.v.                              | 1                                             | 23.5 ± 3.5                                       | <1                                  | <1                                                |
|              |                                    | i.t.                              | 1                                             | 12.9 ± 6.5                                       | <1                                  | <1                                                |
|              |                                    | 10                                | 53.7 ± 12.5                                   |                                                  | <1                                  | <1                                                |
|              | 15                                  | i.v.                              | 1                                             | <1                                              | 23.2                                | 70.8 ± 15.9                                       |
|              |                                    | i.t.                              | 25                                            | 42.0 ± 0.7                                       | <1                                  |                                                   |
|              |                                    | i.t.                              | 1                                             | 37.0 ± 11.6                                      | <1                                  |                                                   |
|              |                                    | 10                                | 30.6 ± 7.8                                     |                                                  | <1                                  |                                                   |
|              | 31                                  | i.v.                              | 25                                            | 289.0 ± 130.9                                    | <1                                  | <1                                                |
|              |                                    | i.t.                              | 1                                             | 29.2 ± 35.2                                      | <1                                  |                                                   |
|              |                                    | 10                                | 17.2 ± 19.8                                    |                                                  | <1                                  |                                                   |
|              | 72                                  | i.v.                              | 50                                            | 543.6 ± 144.8                                    | <1                                  |                                                   |
|              |                                    | i.t.                              | 10                                            | <1                                              | <1                                  |                                                   |
| Mouse        | 9                                   | i.v.                              | 0.100                                         | 15.0 ± 0.0                                       | ND                                  |                                                   |
|              |                                    | i.t.                              | 0.250                                         | <1                                              | ND                                  |                                                   |
|              |                                    | i.t.                              | 0.100                                         | <1                                              | ND                                  |                                                   |
|              |                                    | i.t.                              | 0.075                                         | <1                                              | ND                                  |                                                   |

Irradiated (600–750 rad) M520 (RT-7⁺, IgK-1⁺) rats were injected i.t. or i.v. with varying numbers of histocompatible ALB (RT-7⁺, IgK-1⁺) bone marrow cells. Donor-origin thymocytes (RT-7.2) and splenic B lymphocytes (IgK-1⁻) were quantified by immunofluorescence analysis on the FACS at various days after reconstitution. Results represent the mean ± SD of three animals per group. Values of <1 indicate that no donor-origin hemopoietic spleen colonies, thymocytes, or B lymphocytes were detected. ND, not determined. Hemopoietic spleen colony values were corrected for colonies produced from endogenous stem cells. Irradiated (700 rad) C57BL/6 (Ly 1.2) mice were injected i.t. or i.v. with varying numbers of C57BL/6-Ly 1.1 bone marrow cells. Splenies were removed on day 9 after cell transfer and fixed, and hemopoietic spleen colonies were enumerated.

15), no donor-origin B cells were observed in the spleens of irradiated recipients 15 d after i.t. injection of 10⁶ or 10⁵ rat bone marrow cells. However, >60% (2.32 × 10⁷) of the splenic B cells in recipients of 10⁶ bone marrow cells injected i.v. were of donor origin by day 15. Even by days 31 and 72, no donor-origin B lymphocytes were detected in the spleens of i.t.-injected recipients, whereas permanent B cell chimeras were established in the i.v.-injected recipients.

Absence of Donor-origin Prothymocyte Activity in Bone Marrow of i.t.-injected Recipients. 10⁷ rat bone marrow cells from rats that had received 10⁶ bone marrow cells i.t. or 2.5 × 10⁷ bone marrow cells i.v. 15 d previously were transferred by i.t. injection into secondary recipients (Table V). No donor-origin thymocytes were observed in the secondary recipients given bone marrow from the i.t.-injected donors, whereas 6.98 × 10⁷ donor-origin thymocytes were detected in the secondary recipients given bone marrow from the i.v.-injected donors. Comparable results were observed in the mouse system (Table V).
Tissue Distribution of Cells Capable of Generating Thymocytes in i.t.-injected Recipients. The results in Table VI indicate that, of several hemopoietic tissues examined in both the rat and mouse, bone marrow has the greatest thymocyte-regenerative capacity, on a per-cell basis, and lymph node has the least (if any). Spleen and peripheral blood are intermediate in their capacities to regenerate thymocytes in irradiated recipients.

It is notable that the i.t. injection of $10^7$ rat thymus cells yielded $2.53 \times 10^7$ donor-origin thymocytes by day 14 after transfer, indicating that the i.t. transfer assay readily detects intrathymic as well as prethymic precursor cells. Moreover, results in Fig. 3 show that there is a direct relationship between the number of thymocytes injected i.t. and the number of donor-origin thymocytes detected. The dose-response relationship was linear in the range of $2.5 \times 10^6$–$10.0 \times 10^6$ thymocytes transferred. Results in Fig. 3 also indicate that, when measured at day 14, the i.t. transfer assay is ~53 times more sensitive than is the i.v. transfer system in detecting intrathymic precursor cell activity.

Discussion

Our results show that the i.t. transfer system for thymocyte precursors, like the traditional i.v. transfer system, is quantitative with regard to time and dose and is irradiation dependent. Indeed, when adjusted for the dose of transferred cells, the kinetics of thymocyte and peripheral T cell generation in both assays are roughly comparable, as are the ratios of TdT+ and TdT− thymocyte subsets and T cell subsets with helper and suppressor antigenic phenotypes. However,
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Table VI

Tissue Distribution of Thymocyte Progenitor Activity as Detected by Intrathymic Adoptive Transfer System

| Animal model | Origin of cells transferred | Number of cells transferred ($\times 10^{6}$) | Number of donor-origin thymocytes per thymus ($\times 10^{6}$) |
|--------------|-----------------------------|---------------------------------------------|--------------------------------------------------|
| Rat          | Bone marrow                 | 1                                           | 18.1 ± 5.9                                       |
|              | Thymus                      | 10                                          | 25.3 ± 6.9                                       |
|              | Spleen                      | 5                                           | 13.6 ± 7.9                                       |
|              | Lymph node                  | 10                                          | $<$1                                             |
|              | Peripheral blood            | 5                                           | 14.9 ± 12.9                                      |
| Mouse        | Bone marrow                 | 0.1                                         | 2.5                                              |
|              | Thymus                      | 2.0                                         | 3.2                                              |
|              | Spleen                      | 0.5                                         | 4.3                                              |
|              | Lymph node                  | 5.0                                         | $<$1                                             |
|              | Peripheral blood            | 1.0                                         | 10.2                                             |

Irradiated (600 rad) LEW (RT-7') rats were injected i.t. with histocompatible NBR (RT-7) cells obtained from various lymphoid tissues. Donor-origin thymocytes (RT-7.2) were quantified 14 days after cell transfer by indirect immunofluorescence analysis on the FACS. Results represent the mean ± SD of three animals per group. Values $<$1 indicate that no donor-origin thymocytes were detected. Irradiated (600 rad) C57BL/6 (Ly-1.2) mice were injected i.t. with C57BL/6-Ly 1.1 cells obtained from various lymphoid tissues. Donor-origin thymocytes (Ly-1.1) were quantified 15 d after cell transfer.

Figure 3. The kinetics of appearance of donor-origin thymocytes on day 14 as a function of the dose of NBR (RT-1', RT-7') thymocytes injected i.t. into irradiated (600 rad) LEW (RT-1', RT-7') rats. The dose-response relationship was linear (linear regression coefficient $r^2$ = 0.99; slope = 2.93) in the dose range of $2.5 \times 10^6$–$10.0 \times 10^6$ thymocytes. Recipients injected with $1 \times 10^8$ thymocytes i.v. yielded $9.0 \times 10^6$ donor-origin thymocytes, demonstrating that, at this dose and time point, the i.t. transfer system is 53-fold more sensitive than is the i.v. transfer system at detecting intrathymic precursor cells.
there are three major differences between the i.t. and i.v. assay systems that enable them to address different aspects of thymocytopoiesis. (a) The i.t. assay system is significantly more sensitive than is the i.v. assay system, thereby enhancing the ability to detect both prethymic and intrathymic precursor cells. (b) The i.t. assay system is not dependent upon the ability of thymocyte precursors to migrate to the thymus. This presumably accounts in large part for the increased sensitivity of the i.t. assay, and theoretically should enable precursor cells that have not yet acquired migratory properties and/or that have lost migratory properties to be detected. (c) The i.t. transfer system does not permit the extrathymic engraftment of donor-origin hemopoietic spleen colonies, B lymphocyte progenitors, or prothymocytes in irradiated recipients. This permits the generation of donor-origin thymocytes and T cells to be studied in the absence of continuous seeding of lymphoid precursor cells from the bone marrow. These unique properties of the i.t. transfer system and some implications regarding thymocytopoiesis are discussed briefly below.

Other things being equal, comparison of the relative sensitivities of the i.t. and i.v. transfer systems during the linear phase of the time and dose responses should permit a rough estimate of the seeding efficiency of bone marrow prothymocytes to the thymus after i.v. injection. Thus, assuming that all of the prothymocytes that are injected i.t. survive and proliferate, and assuming that both assays detect the same population(s) of prothymocytes, it can be calculated that the seeding efficiency of mouse prothymocytes in the i.v. assay is 0.04 (1/24). In the rat, this value would be between 0.02 and 0.06, depending on whether or not the relative sensitivity of the i.v. transfer system (1/42 to 1/18) is corrected for the 48-h difference in the appearance of donor-origin cells (see Fig. 1A).

It is possible that the estimated seeding efficiency for prothymocytes in the i.v. assay is too high, because the i.t. transfer system may not be 100% efficient, or too low, because the i.t. transfer system may detect populations of prethymic cells that are unable to home to the thymus. Regarding the latter possibility, we have used the FACS to separate two subsets of rat prothymocytes, both of which generate thymocytes in the i.t. assay, but only one of which does so in the i.v. assay (9, 24, and unpublished observations). In addition, we have observed that prethymic cells in the bone marrow of motheaten (me/me) mice, which undergo premature thymic involution (25), can repopulate the thymus of irradiated normal recipients after i.t. but not i.v. injection. 2

Similar phenomena may also apply to intrathymic precursor cells, some of which are able to proliferate in the i.t. assay but apparently are not able to home to the thymus in the i.v. assay (B. Mathieson, personal communication). This may account in part for the high efficiency of the i.t. transfer system in detecting intrathymic precursor cells.

Numerous reports using the i.v. assay (e.g., 1, 2, 26) have indicated that the relative frequency of thymocyte progenitors in hemopoietic tissues, in order of decreasing frequency, are bone marrow > spleen > thymus > lymph node. We have obtained similar results using the i.t. assay system. In each instance, however,
25–50-fold fewer cells were required in the i.t. assay than in the i.v. assay to generate equivalent numbers of donor-origin thymocytes in the adoptive recipients. It is important that, even given the increased sensitivity of the i.t. assay, lymph node cells were unable to generate donor-origin thymocytes. Hence, it is highly probable that the donor-origin thymocytes detected in the i.t. assay system, including those produced by intrathymic cells, arise from developmentally immature cells and not from the resident pool of immunologically competent cells. In this regard, Fowlkes et al. (10) have used the i.v. transfer system to identify a population of presumptive intrathymic precursor cells that appears to be able to repopulate all of the cellular compartments of the thymus in mice. This is consistent with our observation that the i.t. injection of intrathymic (as well as prethymic) precursors results in the generation of TdT+ and TdT− donor-origin thymocytes, thereby suggesting that both cortical and medullary thymocytes, respectively, are generated in this system as well (19).

As noted, the i.t. assay in the rat and mouse results in the selective and temporary engraftment of thymocyte precursors intrathymically only. No donor-origin hemopoietic colonies or B lymphocytes were detected in the spleen, and no donor-origin prothymocyte activity was detected in the bone marrow of adoptive recipients after the i.t. injection of 10 times more bone marrow cells than was necessary for optimal thymocyte regeneration. In the i.v. assay, however, donor-origin CFU-S, B cell progenitors, and prothymocytes seeded extrathymically, resulting in the establishment of permanent hemopoietic and B and T lymphocyte chimeras.

It can be calculated from these experiments that <1% of the transferred CFU-S, B cell progenitors, and prothymocytes could have become engrafted extrathymically in the i.t. assay system. Therefore, it is evident that the generation of donor-origin thymocytes in the adoptive recipients resulted from the local proliferation of the intrathymically injected precursor cells. Moreover, the results suggest that thymocyte regeneration in the i.t. system is not due to pluripotent stem cells, since the i.t.-engrafted precursors apparently have a limited capacity for self-renewal. Hence, in contrast to the i.v. assay, where continuous generation and seeding of thymocyte progenitors to the thymus occurs, a self-limiting wave of donor-origin thymocytopoiesis occurs in the i.t. assay system.

Exponential regression analysis of the time-response curve has commonly been used to estimate the number of prothymocytes that enter the thymus shortly after the i.v. transfer of bone marrow cells into irradiated recipients (1, 3, 4). However, similar analysis of the kinetics of thymocyte generation in the i.t. assay, in which considerations of seeding efficiency and stem cell chimerism are not germane, show that this statistical approach is unreliable. Thus the y intercept calculated by exponential regression analysis of the data in Fig. 1, A and B suggest that ~25% of the injected rat bone marrow cells and 50% of the injected mouse bone marrow cells were prothymocytes, which is not credible (9). The most probable explanation for this overestimation is the loss of a constant proportion of newly generated thymocytes by intrathymic death or migration to peripheral lymphoid tissues. Thus, the calculated doubling rates in the time response curves of both the i.v. and i.t. assays are low (2.5 d in the mouse; 2.25 d in the rat), and reflect the net accumulation of thymocytes rather than the
gross production of thymocytes. Only the latter data would permit the true y intercept to be calculated, and then only if the doubling time were constant throughout the assay.

One of the characteristic features of thymocytopoiesis in the i.v. transfer system is the 12–14-d lag period that precedes the appearance of donor-origin thymocytes. Although the explanation for this phenomenon is not known (reviewed in 1), Kadish and Basch (3) have provided indirect evidence that the lag is not due to a significant delay in the entry of the transferred prothymocytes into the thymus; migration apparently is completed within the first 48 h after reconstitution. Our results using the i.t. assay, in which the requirement for migration is bypassed entirely, directly excludes this possible mechanism for the lag phase. Moreover, we have previously demonstrated (1) and confirm here that >98% of thymocytes in the RT-7-disparate rat chimeras and the Ly-1-disparate mouse chimeras can be positively identified as being of donor or host origin at all stages of regeneration, thereby excluding the possibility that significant numbers of donor-origin thymocytes escape detection because they fail to express detectable pan-T cell alloantigens during the lag period.

An important feature of the dose-response curve in the i.t. transfer system is the occurrence of a plateau of thymocyte production at peak levels over at least a fivefold range of prothymocytes (see Fig. 2, A and B). This has not been described in the i.v. transfer system, presumably because sufficiently large numbers of bone marrow cells have not yet been transferred experimentally (we estimate that at least $10^8$ cells would be required in the rat). Further analysis of the dose-response curve in Fig. 2A shows that it saturates in an exponential manner and reaches a limiting asymptote of $6.2 \times 10^7$ thymocytes as determined by reciprocal and hyperbolic equations (best fit, $r^2 = 0.97$) (27). This suggests as one possibility that the thymus has a finite number of microenvironmental sites or niches that can support thymocytopoiesis. Moreover, the time-response kinetics (see Fig. 1, A and B) suggest that, once these niches are saturated by prothymocytes, any excess prothymocytes that might have been injected i.t. are not stimulated to proliferate, and apparently are unavailable or unable to refill these sites at a later time; hence, the rapid decrease that occurs in the numbers of donor-origin thymocytes after day 20 in the rat and day 26 in the mouse (see Fig. 1, A and B). Conversely, in the i.v. transfer system, the number of donor-origin thymocytes does not decrease with time, presumably because vacated microenvironmental niches are filled by newly formed donor-origin prothymocytes, which emigrate from the chimeric bone marrow of the host.

In his classical experiments on thymus grafting between age-disparate donors and hosts, Metcalf (28) demonstrated that the thymus functions autonomously to regulate the kinetics of thymocytopoiesis. The i.t. transfer system offers a unique opportunity to explore the thymus microenvironment for this and related phenomena (e.g., “gating,” physiological involution, radiation sensitivity) (1, 3, 27, 28) by separating the purely intrathymic influences on thymocytopoiesis from the prethymic influences (e.g., stem cell chimerism, precursor cell migration). The i.t. transfer system also promises to provide a heretofore unavailable in vivo, organ culture–like environment in which the developmental potential and differentiation pathways of selected prethymic and intrathymic precursor cell sub-
sets and their progeny can be defined. Intrathymic adoptive transfer studies in our and other laboratories currently are addressing these fundamental questions.

Summary

We describe a quantitative intrathymic (i.t.) adoptive transfer system for detecting thymocyte precursor cells in rats and mice. In this system, the generation of donor-origin thymocytes is analyzed on the FACS after the injection of test cells directly into the thymus of sublethally irradiated, histocompatible, RT-7 (rat) or Ly-1 (mouse) alloantigen-disparate recipients. Like the standard i.v. adoptive transfer assays for prothymocytes, the i.t. transfer assay is time, dose, and irradiation dependent. However, unlike the i.v. assays, the i.t. assay is highly sensitive, independent of cell migration, and specific for T-lineage precursor cells. Thus, the i.t. system requires between 25- and 50-fold fewer precursor cells than do the i.v. systems to generate a given number of donor-origin thymocytes; it detects nonmigratory as well as migratory subsets of precursor cells; it detects prethymic and intrathymic precursor cells with equal facility; and it produces a discrete, self-limited wave of donor-origin thymocytes and peripheral T cells. Moreover, neither hemopoietic nor lymphopoietic stem cell chimerism occurs at extrathymic sites.

Comparison of the kinetics of thymocytopoiesis in the i.t. and i.v. transfer systems suggest that the seeding efficiency of prothymocytes in the i.v. assay approximates 0.04; the lag phase of the time-response curve is not due to a delay in the entry of prothymocytes into the thymus; and the relative amount of thymocyte precursor activity in various lymphohemopoietic tissues is highest in bone marrow, lowest (or absent) in lymph node, and intermediate in spleen, blood, and thymus. Moreover, the occurrence of saturation kinetics in the dose-response curve of the i.t. system supports the hypothesis that a finite number of microenvironmental niches for prothymocytes may exist in the thymus. These initial observations will require confirmation and extension in future studies. However, based on the present findings and related observations, we anticipate that the i.t. adoptive transfer system will contribute importantly to the definitive analysis of both normal and abnormal thymocytopoiesis.

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