*Proc. Nat. Acad. Sci. USA*
Vol. 71, No. 8, pp. 3124–3128, August 1974

# Selective Thymic Localization of Murine Leukemia Virus-Related Antigens in C57BL/Ka Mice after Inoculation with Radiation Leukemia Virus

**(immunofluorescence/thymus/bone marrow)**

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*Contributed by Henry S. Kaplan, May 16, 1974*

**ABSTRACT** **The tissue distribution and extent of virus-specific antigen expression were studied by immunofluorescence as a function of time and of lymphoma development in adult C57BL/Ka (Fv-1$^b$) mice after intravenous injection of radiation leukemia virus, a B-tropic murine leukemia virus. Viral antigens were detected earlier in the thymus (1 week) than in the bone marrow, spleen, or lymph nodes (2–3 weeks). Despite an initial virus-induced thymic involution, the percentage of immunofluorescence-positive cells in the thymus rapidly increased thereafter to 65–80%, at which level it remained until 9 weeks, at which time increases in size and weight, histological changes, and an increased number of blastoid cells indicated the onset of lymphoma development in the thymus. In contrast, the percentage of immunofluorescence-positive cells in the bone marrow, spleen, and nodes remained low, and gradually decreased to zero within 8 weeks after thymectomy. The selective thymic localization of antigens induced by radiation leukemia virus in C57BL/Ka mice is in striking contrast to the previously reported ubiquitous tissue distribution of the Gross-AKR virus, an N-tropic virus, in its natural host, the Fv-1$^n$, AKR strain with a high incidence of leukemia.**

The many murine leukemia viruses (MuLV) isolated (for review see ref. 1) may be classified into two groups: (*a*) a naturally occurring group originally extracted from leukemias and lymphomas of the murine strains in which they are "vertically" transmitted; and (*b*) a "virulent" group, originally extracted from long-transplanted murine neoplasms of non-lymphomatous character and not known to exist as such in nature (2). The rate of propagation of different MuLV on cultured cells derived from different murine strains is determined by the Fv-1 gene (3, 4). Certain naturally occurring viruses replicate to titers $10^2$- to $10^3$-fold higher on the cells of Fv-1$^n$ strains (NIH, AKR, $C_3H$, etc.) than on those of Fv-1$^b$ strains (BALB/c, C57BL, etc.); such viruses are designated "N-tropic." Conversely, other naturally occurring viruses exhibit the reciprocal pattern of preferential replication on Fv-1$^b$ cells and are designated as "B-tropic."

Although much has now been learned concerning their behavior *in vitro*, relatively little information is available concerning the time-course of expression and patterns of tissue spread *in vivo* of the naturally occurring group. It is known that one of the prototype N-tropic viruses, the Gross-AKR wild-type virus, first becomes detectable in infectious form at about the time of birth in its natural host, the AKR strain (5, 27). Within a few days thereafter, it is widely distributed in spleen, liver, bone, bone marrow, and other tissues, including the thymus, in which the lymphomas it induces are known to arise. The rapidity with which Gross-AKR virus becomes ubiquitously disseminated in infant AKR mice seemed entirely consistent with expectation for an N-tropic virus in a host of Fv-1$^n$ genotype, and presumably related to the high "spontaneous" incidence of leukemia in AKR mice (6).

It seemed important to investigate the pattern of tissue distribution *in vivo* of a B-tropic virus in a host of Fv-1$^b$ genotype. The B-tropic MuLV selected for these studies was the radiation leukemia virus (RadLV), originally extracted from thymic lymphomas induced in strain C57BL/Ka mice (which have a low incidence of leukemia) by whole-body x-irradiation and known to be leukemogenic in mice of that strain when inoculated intraperitoneally, intravenously, or intrathymically soon after birth (7–9). Unlike Gross-AKR leukemia virus, the endogenous virus is not spontaneously expressed in infectious form until very late in life (10, 11); before that time, its expression may be induced by exposure of C57BL/Ka mice to appropriate doses of whole-body x rays (7, 8) or certain chemicals (12–14). Accordingly, it was elected to inject exogenous RadLV intravenously (i.v.) into young adult C57BL/Ka mice and to follow the pattern and relative abundance of viral antigen expression in selected lymphoid and hematopoietic tissues as a function of time and of lymphoma development.

## MATERIALS AND METHODS

*Mice.* Female C57BL/Ka mice were used. They were 40–45 days old at the start of all experiments.

*Virus Preparations.* RadLV preparations were cell-free extracts of RadLV-induced C57BL mouse lymphoma tissue, prepared as described (7, 15). Virus was assayed by *in vitro* (16, 17) and *in vivo* (intrathymic injection)* methods. Titers ranged from $2 \times 10^5$ to $2 \times 10^6$ infectious units per 0.4 ml.

*Inoculation of Mice.* Undiluted RadLV mixed with 5–10 units/ml of heparin was injected i.v. in a volume of 0.2 ml. In one experiment, serial dilutions of the virus were used.

*Preparation of Cell Suspensions.* Mice were killed with ether. Thymus, spleen, and mesenteric lymph nodes were dissected free from adjacent tissues and weighed. Lymphocyte suspensions were prepared by mincing each tissue, then passing the suspension through a nylon cloth into phosphate-buffered saline (pH 7.4). Cells were counted to determine the

Abbreviations: MuLV, murine leukemia virus; RadLV, radiation leukemia virus; i.v., intravenous; IF, immunofluorescence.

* A. Declève, M. Lieberman, O. Niwa, and H. S. Kaplan, manuscript submitted for publication.

TABLE 1. *Percentage of IF-positive cells in the thymus of C57BL mice at different times after injection of serial dilutions of RadLV*

| Virus dilutions | Weeks after RadLV injection (i.v.) 1 | 2 | 3 |
|---|---|---|---|
| Undiluted | 12.0* | 62.0 | 80.0 |
| 1:5 | 0.05 | 24.5 | 87.0 |
| 1:25 | 0.00 | 4.2 | 74.0 |
| 1:125 | 0.00 | 0.02 | 50.0 |
| 1:625 | 0.00 | 0.00 | 9.0 |
| 1:3125 | 0.00 | 0.00 | 6.0 |
| 1:15625 | 0.00 | 0.00 | 0.00 |
| Controls | 0.00 | 0.00 | 0.00 |

* Percentage of IF-positive thymic cells at the 1:16 and 1:32 dilutions of rat antiserum against MuLV. Values given are the means of 2–4 mice per point; 10,000 to 20,000 thymic cells per mouse scored at higher dilutions. Normal serum and phosphate-buffered saline controls revealed no IF-positive thymic cells.

total number of cells in thymus and bone marrow, and cell concentrations were adjusted to levels suitable for the assay of percent immunofluorescence (IF).

*IF Test for Detection of MuLV Antigen.* Indirect immunofluorescence staining of acetone-fixed cells was carried out as described by Hilgers *et al.* (18). The immune and fluoresceinated antisera have been described (17). The percentage of fluorescent cells was counted over a range of dilutions of rat virus-specific immune sera. In addition to three dilutions of a given immune serum, each slide included normal serum and phosphate-buffered saline controls. Values presented for "% MuLV antigen-positive cells" are the means of three data points in the plateau region of the serum dilution curve (1:16 to 1:32) and indicate the maximal proportion of viral antigen-containing cells in each preparation. For each point, 10,000–20,000 cells were counted.

*Thymectomized Animals.* Groups of mice were thymectomized by a previously described procedure (19) 7 days before virus inoculation.

*Differential Cell Count.* Cells were mixed with Turk's solution for differential cell counting in the hemocytometer. Smears were counted after May–Grünwald–Giemsa staining. Small lymphocytes had a nucleus less than 7 $\mu$m in diameter in the smears and 4.5 $\mu$m in the hemocytometer. Medium and large blastoid lymphocytes were much larger, and could be distinguished from reticulum cells by virtue of their round nucleus, basophilic cytoplasm, and high nuclear-to-cytoplasmic ratio.

## RESULTS

*Detection of Viral-Related Antigens in the Thymus of C57BL Mice after i.v. Injection of Serial Dilutions of RadLV.* The titer of RadLV stock used for this experiment was estimated at 2 to 4 $\times$ $10^6$ infectious units per ml. Table 1 shows the percentage of IF-positive cells in the thymus at 1, 2, and 3 weeks after injection of serial 5-fold dilutions of RadLV. It is clear that only high concentrations of RadLV (undiluted or 1:5 dilutions) yielded detectable amounts of virus-related antigens as early as 1 week after i.v. injection. At 3 weeks, the end point dilution yielding IF-positive cells was 1:3125.

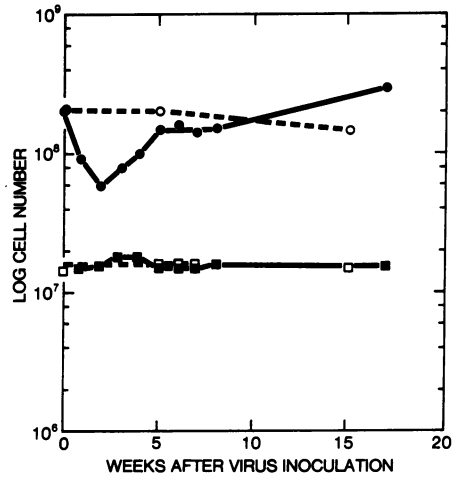


FIG. 1. Total cell count in thymus and bone marrow at various time intervals after intravenous injection of RadLV (undiluted; titer 2 $\times$ $10^6$ infectious units/0.4 ml) or of phosphate-buffered saline into C57BL mice. (○) Thymus of mice injected with phosphate-buffered saline; (●) thymus of RadLV-injected mice; (□) bone marrow of mice injected with phosphate-buffered saline; (■) bone marrow of RadLV-injected mice.

*Total Cell Number in the Thymus and Bone Marrow after i.v. Injection of RadLV in C57BL Mice.* Undiluted RadLV (2 $\times$ $10^6$ infectious units per ml) was injected intravenously into mice. At various times thereafter, the thymus was removed and weighed, and cell suspensions were made from the thymus and bone marrow. Total thymus and bone marrow cell numbers of virus-injected animals and controls injected with phosphate-buffered saline are presented in Fig. 1. Curves for thymus weight and total cell number followed identical patterns. The animals injected with phosphate-buffered saline showed an initial thymic count of 2 $\times$ $10^8$ cells, followed by a slight decrease after 5 weeks with the onset of natural thymic involution.

The virus-injected animals showed a sharp decrease in total thymus cell number, with the nadir value occurring 2 weeks after injection. Total cell number returned to control values by 5 weeks and remained at that level until 9 weeks after injection, after which time it increased progressively, indicating that a preleukemic stage had developed. In four replicate experiments, it was found that the decrease in total thymus cell count was delayed in onset when a more dilute or less potent virus preparation was inoculated. The occurrence of a sharp decrease in thymus weight and cell number after MuLV inoculation has been reported (20–22). In contrast, both the virus-injected animals and those injected with phosphate-buffered saline displayed a constant bone marrow cell count throughout the experiment.

*Percentage of IF-Positive Cells in Lymphoid Organs of C57BL Mice at Different Times after RadLV Inoculation.* The results of four replicate experiments are given in Table 2. Undiluted RadLV with an estimated titer of 2 $\times$ $10^6$ infectious units per ml was used in all except experiment no. 4, in which the titer was 10-fold lower. For each organ, the percentage of positive cells found with phosphate-buffered saline or normal serum (nonspecific fluorescence) is listed in the control column; the percentage found with rat anti-MuLV immune serum is listed in the experimental column. The percentage of cells fluorescing nonspecifically in the absence of anti-MuLV serum was in the

TABLE 2. *Time course of appearance of virus-related antigens in various hematopoietic tissues after intravenous RadLV inoculation in strain C57BL mice*

| | Time after virus inoculation | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 days | | 4 days | | 1 week | | 2 weeks | | 3 weeks | | 4 weeks | |
| | *C* | *E* | *C* | *E* | *C* | *E* | *C* | *E* | *C* | *E* | *C* | *E* |
| *Thymus* | | | | | | | | | | | | |
| Exp. 1 | 0.00 | 0.00 | 0.00 | 0.2* | 0.00 | 9.7 | 0.00 | 37.0 | 0.00 | 68.0 | 0.00 | 80.0 |
| Exp. 2 | — | — | — | — | 0.00 | 10.3 | 0.00 | 64.0 | 0.00 | 75.0 | 0.00 | 63.0 |
| Exp. 3 | — | — | — | — | 0.00 | 12.0 | 0.00 | 62.0 | 0.00 | 80.0 | — | — |
| Exp. 4 | — | — | 0.00 | 0.00 | 0.00 | 0.5 | 0.00 | 2.0 | 0.00 | 35.0 | 0.00 | 56.0 |
| *Bone marrow* | | | | | | | | | | | | |
| Exp. 1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.15 | 0.00 | 0.35 |
| Exp. 2 | — | — | — | — | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.40 | 0.00 | 0.40 |
| Exp. 3 | — | — | — | — | 0.00 | 0.02 | 0.00 | 0.35 | 0.00 | 2.5 | — | — |
| Exp. 4 | — | — | — | — | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.50 | 0.00 | 0.33 |
| *Spleen* | | | | | | | | | | | | |
| Exp. 1 | — | — | — | — | — | — | — | — | — | — | — | — |
| Exp. 2 | — | — | — | — | 2.0 | 2.5 | 1.5 | 3.1 | 2.0 | 8.2 | 2.3 | 7.0 |
| Exp. 3 | — | — | — | — | 2.0 | 0.95 | 1.2 | 2.5 | 1.8 | 9.3 | — | — |
| Exp. 4 | — | — | — | — | 0.5 | 0.4 | 0.8 | 0.8 | 0.4 | 4.6 | 0.35 | 5.0 |
| *Lymph nodes* | | | | | | | | | | | | |
| Exp. 1 | — | — | — | — | — | — | — | — | — | — | — | — |
| Exp. 2 | — | — | — | — | 0.5 | 0.3 | 0.15 | 2.7 | 0.8 | 4.5 | 0.5 | 5.3 |
| Exp. 3 | — | — | — | — | 0.15 | 0.15 | 0.15 | 2.2 | 0.3 | 7.0 | — | — |
| Exp. 4 | — | — | — | — | 0.20 | 0.20 | 0.10 | 0.2 | 0.10 | 2.0 | 0.06 | 0.8 |

* Values are % IF-positive cells. *C* = phosphate-buffered saline or normal-serum controls; *E* = rat anti-MuLV immune serum. —, not done.

range of 0.5 to 2.3 for spleen and less than 0.5 for lymph nodes. Bone marrow cells have occasionally been positive in some experiments at a level of 0.1–0.2%. Similar percentages of positive cells were found in spleen and lymph nodes of mice injected with phosphate-buffered saline after the cell suspensions were treated with anti-MuLV serum, as well as in normal serum controls, indicating clearly that this fluorescence is nonspecific and presumably due to crossreaction of some immunoglobulin-containing lymphoid cells with fluoresceinated goat anti-rat globulin (18).

After virus injection, IF-positive cells were detected in the thymus as early as 4 days, and were consistently present after 1 week. In one experiment a small number of positive cells (2 in 10,000 cells) were detected in the bone marrow one week after RadLV injection. However, significant levels of IF-positive bone marrow cells were not consistently seen until 3 weeks after injection. Their abundance at that time (0.15–2.5%) was very low when compared with that found in the thymus (35–80%). A similar interval was required before a significant increase over control levels could be detected in the percentages of IF-positive cells in spleen and lymph nodes.

TABLE 3. *Percentage of IF-positive cells (% IF) in bone marrow of thymectomized mice at different times after RadLV inoculation*

| Time (weeks) | % IF |
|---|---|
| 1 | 0.028 |
| 2 | 0.004 |
| 3 | 0.028 |
| 4 | 0.10 |
| 6 | 0.015 |
| 8 | 0.00 |

An approximately 4-fold increase was observed in spleen and a 10- to 20-fold increase in lymph nodes. At 3 weeks, the control mice injected with phosphate-buffered saline still had the normal background level of nonspecifically fluorescing cells (about 2% in the spleen and 0.2% in the lymph nodes) whether the cells were treated with rat anti-MuLV serum or normal serum.

Figure 2 shows the percentage of positive cells in the thymus and bone marrow at serial intervals up to 17 weeks after RadLV injection (titer $2 \times 10^6$/0.4 ml). In the thymus, the response reached a plateau level of 60–80% positive cells within 2–3 weeks after inoculation in two replicate experiments. The bone marrow reached a plateau level of less than 1% by 3–4 weeks after inoculation. The plateau remained almost constant thereafter in both organs, increasing only slightly toward the end of the 17-week observation period.

*Percentage of Blastoid Cells in the Thymus and Bone Marrow after RadLV Inoculation.* In the experiment described in Fig. 2, the relative number of blastoid cells was also followed in bone marrow and thymus cell suspensions. Fig. 3 shows that their percentage in bone marrow remained constant at about 30% in both RadLV-injected animals and those injected with phosphate-buffered saline throughout the experiment. In contrast, there was a striking virus-induced increase in blastoid cells in the thymus. Two weeks after virus inoculation, the percentage of blastoid cells was twice that seen in the controls injected with phosphate-buffered saline. By 3 weeks after injection, an initial plateau of about 30% was reached which was maintained for 5 more weeks. At 9 weeks, the number of blastoid cells increased again to reach about 80% at the end of the observation period (17 weeks), concomitantly with the first appearance of frank lymphomas in the virus-inoculated mice.

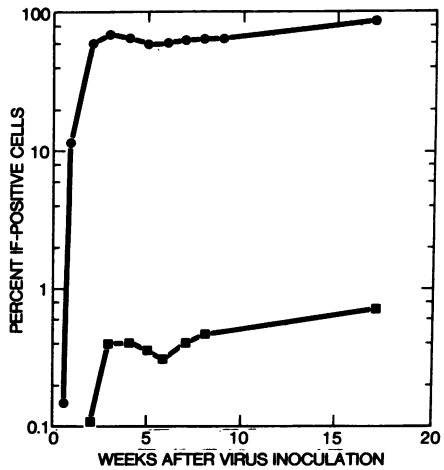


FIG. 2. Percentage of IF-positive cells in thymus and bone marrow of C57BL mice at various time intervals after intravenous injection of undiluted RadLV ($2 \times 10^6$ infectious units/0.4 ml). (●) Thymus; (■) bone marrow.

*Total IF-Positive Cell Count in the Thymus of RadLV-Injected Animals.* To estimate the total number of positive cells in the thymus at each interval, we multiplied the total cell count (Fig. 1) by the percentage of positive cells (Fig. 2), and plotted the resulting value against time in weeks after inoculation (Fig. 4). The total number of cells in which virus-related antigens can be detected increased dramatically despite the initial decrease in total cell number. By 5 weeks after inoculation, almost every lymphoid cell in the thymus had become IF-positive, and all cells remained positive through the ninth week, at which time the thymus showed distinctive signs of early lymphoma development (weight and size increase, histology, and increase in the percentage of blastoid cells).

*Virus-Related Antigen Expression in the Bone Marrow of Thymectomized RadLV-Injected C57BL Mice.* Since the bone marrow and spleen become IF-positive in RadLV-injected C57BL mice only after the thymus does, it is reasonable to postulate that cells detected as positive in the bone marrow and spleen migrate there from the thymus after viral antigen expression has occurred. Alternatively, bone marrow and spleen cells may leave those organs and migrate to the thymus very soon after viral antigen expression occurs in them. In the former case, and perhaps also in the latter, positive cells should not accumulate in these tissues in thymectomized mice. The results shown in Table 3 indicate that when the thymus was removed, the percentage of positive cells in the bone marrow rose no higher than 0.1% at 4 weeks and decreased thereafter. By 8 weeks after inoculation, not a single fluorescent cell could be found among 10,000 bone marrow cells scanned.

## DISCUSSION

Studies of the time course of appearance and tissue distribution of Gross-AKR leukemia virus during the development of leukemia in the AKR strain of mice with a high incidence of leukemia revealed that infectious virus appeared and increased rapidly to high titer within the first week after birth (5). In 5- to 6-week-old animals, practically all tissues contained infectious virus; paradoxically, however, bone marrow, spleen, and lymph nodes had higher titers than thymus. In experiments with $C_3H/Gs$ mice and Gross passage

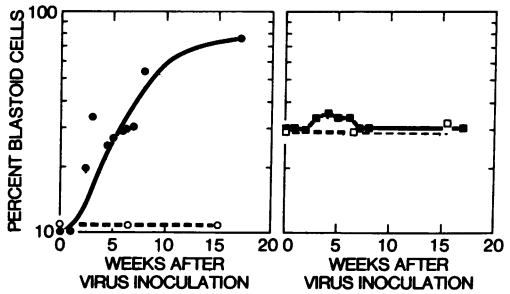


FIG. 3. Percentage of blastoid lymphocytes in the thymus and bone marrow of C57BL mice at various time intervals after intravenous injection of phosphate-buffered saline or RadLV (titer, $2 \times 10^6$ infectious units/0.4 ml). (○) Thymus of mice injected with phosphate-buffered saline; (●) thymus of RadLV-injected mice; (□) bone marrow of mice injected with phosphate-buffered saline; (■) bone marrow of RadLV-injected mice.

A virus, Miller (23) concluded that the leukemogenic agent could be recovered from the tissues of inoculated mice for 6 months or more after thymectomy, that replication of the leukemogenic agent could take place during serial transfer through thymectomized hosts, and that the thymus is not a necessary site for replication of the virus.

In striking contrast, the present experiments reveal that RadLV, a B-tropic virus, does not become ubiquitously established in high titer in tissues other than the thymus after intravenous injection into young adult mice of its strain of origin, C57BL/Ka ($Fv\text{-}1^b$). Virus-specific antigens are detectable several days earlier in the thymus than in spleen, lymph nodes, or bone marrow, and the proportion of IF-positive cells in the latter tissues never rises to levels comparable to those observed in the thymus.

Thymocytes become positive for virus-related antigens long before any signs of transformation are discernible. By the time the percentage of blastoid cells begins to increase, 10%

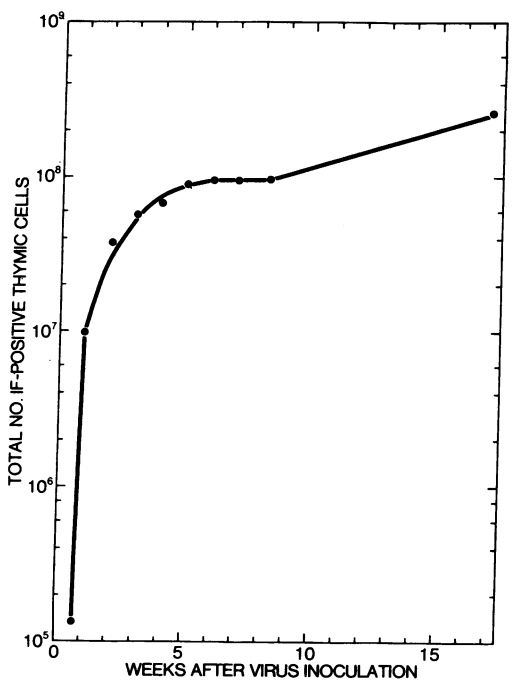


FIG. 4. Total number of IF-positive cells in the thymus of C57BL mice at various time intervals after RadLV inoculation.

of the thymocytes are IF-positive. The very significant decrease in the total number of cells in the thymus after virus inoculation confirms previous observations by others (20–22). It is noteworthy that, despite the decrease in total cell number, the number of IF-positive cells in the thymus increases continuously, indicating that once viral infection has started, there is no apparent "switching off" of virus production. Within 2–3 weeks after infection, 70–80% of the thymocytes are involved in the replication of at least part of the virus. This activity persists until about 9 weeks, at which time the thymus exhibits preleukemic changes associated with a further increase in the proportion of blastoid lymphocytes.

Recently, it has been shown that C57BL bone marrow cells can be infected by RadLV *in vitro* and can undergo lymphomatous transformation *in vivo*, provided that the thymus is present†. In the present paper, we demonstrate that only a very low percentage of C57BL bone marrow and spleen cells *in situ* develop virus-related antigens indicative of their infection by and at least partial replication of the virus. However, radiobiologic studies (24, 25) have clearly established that bone marrow-derived and spleen-derived cells can migrate to the thymus and there apparently undergo metaplastic conversion to cells that are morphologically and functionally indistinguishable from thymocytes. It is therefore not unlikely that bone marrow and spleen cells, as well as thymus cells, were infected by the virus at the time of inoculation, but that the viral infection did not progress to the point of virus-specific cytoplasmic antigen production except in those cells that migrated from the bone marrow and spleen to the thymus. When the thymus was removed, the percentage of IF-positive bone marrow cells decreased to zero by 8 weeks. Thus, it may be concluded that the C57BL thymus must be present not only to allow neoplastic transformation of virus-infected lymphocytes, as previously demonstrated (26), but also to provide an environment essential for the sustained vegetative replication of wild-type RadLV *in vivo*.

No decrease in number of total bone marrow cells comparable to that observed in the thymus occurs after RadLV injection. The delayed appearance of IF-positive cells in the bone marrow and their progressive decrease in number after thymectomy both suggest that cells migrating to the bone marrow from the thymus account for at least part of the IF-positive cell population found in that tissue. The finding that viral antigens were not detected in spleens and lymph nodes until about three weeks after RadLV inoculation, long after the thymus started to be positive, suggests that at least part of the IF-positive spleen and lymph node lymphocyte population is also thymus-derived.

No precise estimate of the maximal initial pool size of the virus-susceptible thymic cell population can be made by extrapolation to time zero in Fig. 4 because (*i*) many cells arise and die *in situ* in the normal thymus; moreover, after virus infection a particularly striking loss of thymic cells is observed (*ii*) the thymus is an open system with many cells moving in and out; (*iii*) there may be a lag phase in cell growth after infection by RadLV; and (*iv*) there may also be a lag phase for the expression of viral antigens.

Estimates of the maximal size of the pool of cells susceptible to RadLV infection would also require injection of saturating concentrations of virus. Even with the most potent RadLV preparations used in these experiments, a saturation plateau could not be demonstrated at 2 weeks after intravenous inoculation of the virus (Table 1). Accordingly, one can state only that $10^5$ cells or less comprised the original pool of infected cells that gave rise to the IF-positive population later observed in the thymus.

This work was supported by Grants CA 03352 and CA 10372 from the National Cancer Institute, National Institutes of Health. C.S. was an Eleanor Roosevelt Cancer Research Fellow of the International Union Against Cancer, on leave from the Aichi Cancer Center, Nagoya, Japan. The excellent technical assistance of Olja Finn and Margo Peacock is gratefully acknowledged.

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