STUDIES ON THE LIFE HISTORY OF LYMPHOCYTES

I. The Life-Span of Cells Responsive in the Mixed Lymphocyte Interaction*

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A series of studies has provided considerable evidence that the mixed lymphocyte interaction (MLI)[1] with rat cells can be a useful model for investigating the initial steps in the immune response to major transplantation antigens (H antigens) (1–6). The specificity, kinetics, and cell numbers involved in the reaction have been demonstrated (2, 3), as well as the fact that most of the lymphocytes involved in the response are of thymic origin (6). One finding, that the proportion of antigen-reactive cells (ARC) to H isoantigens is large and is not significantly increased by immunization, suggests that immune responses to major transplantation antigens may be unique in some respects compared to reactions to other types of antigens (3, 5).

The present studies were designed to extend our knowledge of the life history within the animal of those lymphocytes responsive to H isoantigens (hereafter called H-ARC) with particular emphasis on the life-span of these cells. Through the use of in vivo labeling with tritiated thymidine and subsequent examination of both peripheral blood smears and mixed lymphocyte reactions for labeled cells, the following questions about H-ARC were investigated: (*a*) Are H-ARC part of a "short-lived" or a "long-lived" lymphocyte pool; or more precisely, are these cells which have been recently produced in the intact animal through single or multiple divisions; or, by contrast, are these cells which have survived for a long period in vivo (with or without occasional divisions)? (*b*) Regardless of whether H-ARC are short-lived or long-lived in vivo, are they recently derived from the thymus or can they be produced at extrathymic sites, perhaps by cells themselves derived from the thymus? (*c*) Are H-ARC a radiosensitive cell population?

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[1] *Abbreviations used in this paper*: ARC, antigen-reactive cells; H-ARC, lymphocytes responsive to H isoantigens; ^{3}H-TdR, tritiated thymidine; MLI, mixed lymphocyte interaction; PHA, phytohemagglutinin.

The results indicate that some H-ARC have a long life-span in vivo without division, but that most undergo multiple divisions at frequent intervals, especially in young animals. They need not have recently emigrated from the thymus, but they do appear to be radiosensitive. From these results we suggest a model encompassing several phases of the natural "life history" of H-ARC in the rat, as well as of other peripheral blood lymphocytes, which may be of value in suggesting further studies designed to understand the mechanisms whereby the large number of H-ARC is generated, the function of these cells in the economy of the animal, and their possible role in the ontogeny of the immune response.

Materials and Methods

Rats.—The rats used in this study for in vivo labeling and as cell donors for the mixed lymphocyte interactions were young adults of the highly inbred DA strain, 1–4 months of age. In some experiments cells were also obtained from F_1 hybrids derived from matings of DA and Lewis (L) strains or DA and Brown Norway (BN) strains, which differ from the DA at the major H isoantigen locus (AgB).

Thymectomy.—Thymectomy was performed at 5 wk of age on a number of the DA rats, and they were then used as experimental animals 1 month later. *Sublethal irradiation* (300 rads to each side, 200 kvp X-ray) was also administered to some animals which had been thymectomized or sham-operated 7 days previously; these animals were allowed to recover for 7 wk before experimental use.

Labeling.—Labeling in vivo was carried out by the intraperitoneal injection of tritiated thymidine, 0.7–1.0 μCi/g body weight per injection (specific activity = 6.7 Ci/mM), administered over a period of 1 day (five injections in 8 hr), 4 days (one or two injections per day), or 10 days (three injections per day). Blood smears were made from a tail vein on various days after in vivo labeling and fixed in 70% alcohol.

Radioautographs.—Radioautographs were prepared from the smears by the dipping method with Kodak NTB2 emulsion. The slides were exposed for 4 wk in light-tight boxes at 4°C, after which they were processed in Kodak D19 developer, Rapid Fixer, and water rinses. The cells were then stained through the emulsion with Giemsa stain. Slides from the various experiments were randomized before dipping and scored without the observer's knowledge of their origin.

The labeling index and grain counts for small lymphocytes were determined on at least 100 cells/slide and at least two slides per rat per day. A small lymphocyte was arbitrarily defined as a cell with a diameter equal to or less than one and a half times that of adjacent red cells, and it was considered labeled if the grain count was at least twice that of comparable adjacent red cell areas. In nearly all slides, this resulted in only cells with more than three grains being counted as labeled; although some lightly labeled lymphocytes were undoubtedly omitted under this criterion, concern over labeling due to isotope reutilization was also minimized.

Mixed lymphocyte cultures were established with peripheral blood lymphocytes from labeled parental strain donors and unlabeled F_1 donors by procedures described in detail elsewhere (1, 3). Blood was collected by cardiac puncture at various times after labeling, leaving the donors alive for subsequent study. Leukocyte suspensions, consisting mostly of lymphocytes, were prepared by dextran sedimentation, the cells washed, suspended in medium, and cultured in duplicate or triplicate at a concentration of 2 million/ml, 1 million from each of the two donors.

Proliferation in the cultures was assessed by the incorporation of tritiated thymidine

(^{3}H-TdR) over a 16 hr period with appropriate controls to account for the contribution of label incorporated in vivo. The cultures were terminated at various times and the activity measured by liquid scintillation spectrometry.

Proportion of labeled cells.—The proportion of labeled cells proliferating in the MLI was assessed by determining the percentage of labeled mitotic figures in radioautographs prepared from the cultures. At various times (2–5 days), after initiating the cultures, air-dried slides were prepared from duplicate cultures according to standard techniques for chromosome preparations (7), except that the drying period was prolonged so as to minimize spreading of the mitotic figures. All cultures contained colchicine (final concentration 5×10^{-6} M) throughout the culture period, so that only "first division" mitoses were observed.

Preliminary experiments indicated (*a*) that this concentration of colchicine had no adverse effects on the cultured lymphocytes except to arrest dividing cells in metaphase, (*b*) that after 8–12 hr, arrested cells disintegrated without demonstrable reutilization of their label by others in the culture, and (*c*) that no cells escaped colchicine arrest. Thus, at any time during the culture period, mitoses observed on the slides represented only cells which had entered their first mitotic division in culture during the previous 8–12 hr.

Radioautographs were made by the dipping method as described for the blood smears and stained with Giemsa. A mitosis was considered labeled if the grain count was at least twice that of adjacent background areas, and in practice, this generally meant cells with greater than six grains. Two slides were made from each of the duplicate cultures, and, as with the blood smears, the slides were coded, randomized, processed in batches, and the code was not broken until after the slides were scored.

RESULTS

Labeling Profile of Small Lymphocytes in the Peripheral Blood.—Figs. 1 and 2 and Table I present data from typical experiments on the percentage of labeled small lymphocytes in the peripheral blood at various times after 1, 4, or 10 days of multiple injections with tritiated thymidine.

The percentage of labeled small lymphocytes in the peripheral blood reached a peak of 10–15% by 3–6 days after a single day of labeling injections; thereafter it declined rapidly to levels of 2–5% by 3–4 wk (Fig. 1). Injecting rats with labeled thymidine for 4 days or for 10 days generally resulted in somewhat higher levels of labeled small lymphocytes in the blood, both shortly after injection and 3–5 wk later, but the effect was never additive as compared to animals injected for only 1 day (see Fig. 2 and Table I).

Grain count data are also indicated in Figs. 1 and 2 and Table I. With both single-day and multiple-day labeling, median grain counts over the labeled lymphocytes in the blood reached peak levels very promptly, then declined to less than half the maximum values within a wk after labeling. Thereafter, a very much more gradual decrease was noted. Several factors may contribute to the shape of this curve. The rapid decline in the grain count during the first 3–4 days after labeling could be the result of rapid divisions of the labeled cell population and/or disproportionate loss of heavily labeled cells from the circulation. At later times after labeling, the gradual decline in grain count could reflect occasional single divisions among the remaining labeled lymphocyte population. The latter possibility is supported by the fact that in several

experiments many labeled cells at 3–5 wk had grain counts below the minimum values observed shortly after labeling (see Table I).

Thymidine injections extending over a 4 or 10 day period had the effect of increasing the median grain count of labeled lymphocytes in the blood during both the early and late postlabeling periods. Blood smears from animals in-

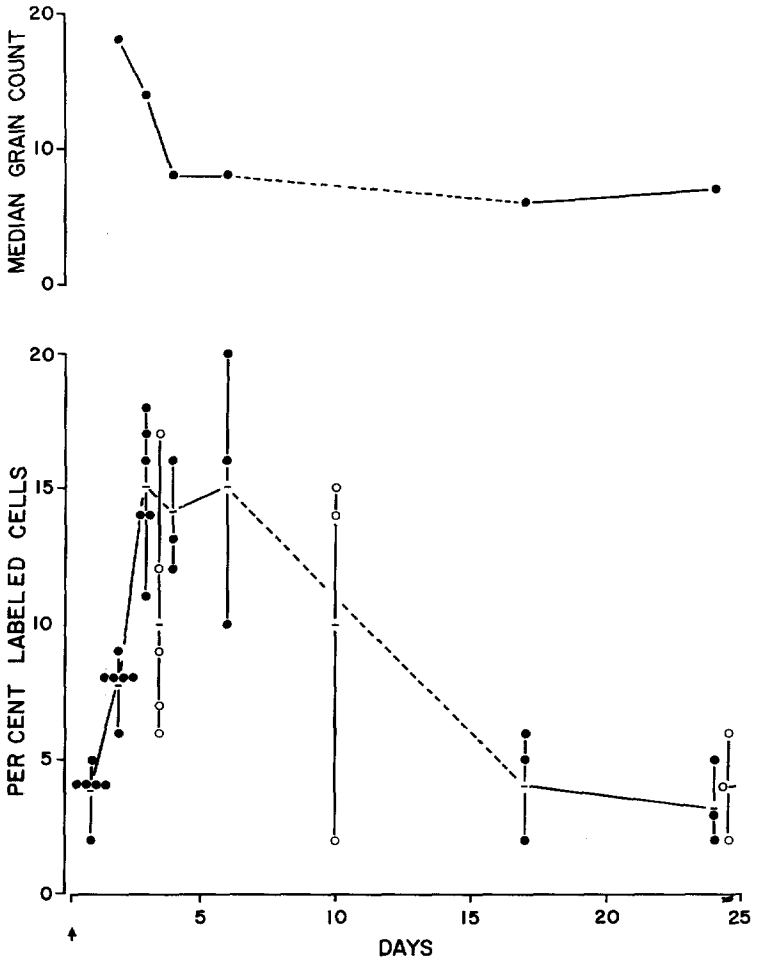

FIG. 1. Labeling profile of peripheral blood small lymphocytes and of mitoses (H-ARC) in MLI from rats injected with ^{3}H-TdR for 1 day. *Upper Graph*: Grain count data on peripheral blood small lymphocytes (each point represents the mean of median grain counts from six rats). *Lower Graph*: Per cent labeled lymphocytes in blood (●) and per cent labeled mitoses in MLI (○). Each point = one rat (at least 200 lymphocytes or 100 mitoses); (—) = mean of values; (↑) = day of ^{3}H-TdR injection.

jected for only 1 day rarely had lymphocytes with more than 50 grains, but such cells were common shortly after multiple-day labeling, and a few were still present 3–5 wk later (Table I).

The fact that injecting ^{3}H-TdR over several days in comparison to a single day did not produce a marked additive increase in the total number of labeled

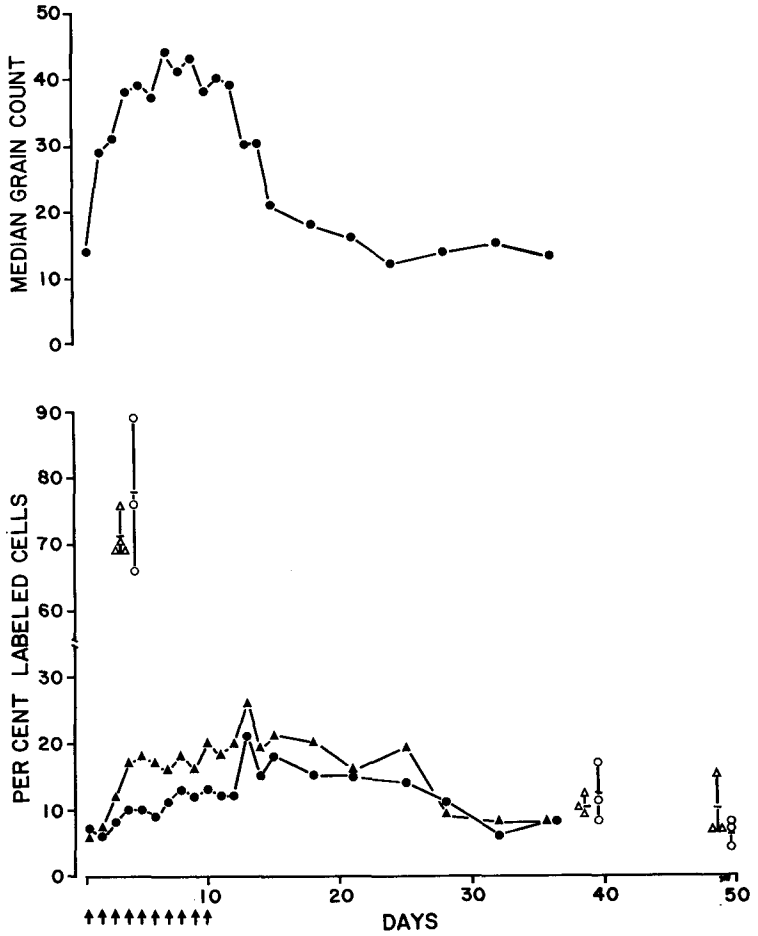

FIG. 2. Labeling profile of peripheral blood small lymphocytes and of mitoses (H-ARC) in MLI from thymectomized and sham-operated rats injected with ^{3}H-TdR for 10 days. *Upper Graph*: Grain count data on peripheral blood small lymphocytes (each point represents the mean of median grain counts from seven rats: four thymectomized, three sham-operated). *Lower Graph*: Per cent labeled lymphocytes in blood (▲—▲) = thymectomized, (●—●) = sham-operated. Each point = mean of three to four rats; and per cent labeled mitoses in MLI (△) = thymectomized, (○) = sham-operated. Each point = one rat; (—) = mean of values; (↑) = days of ^{3}H-TdR injections.

lymphocytes, but did increase the median grain count, suggests that most of the lymphocytes subsequently scored as labeled cells in the peripheral blood were in a proliferative phase involving rapid multiple sequential divisions during the time that label was available to them. This appears true of long-lived lymphocytes as well as those observed within a few days after labeling.

Adult Thymectomy.—The effect of adult thymectomy on the labeling profile of small lymphocytes in the blood was also investigated. (Fig. 2 and Table I, Fig. 3 and Table III). In rats injected with ^{3}H-TdR at least 30 days post-

TABLE I

Labeling Profile of Small Lymphocytes in Peripheral Blood of Thymectomized and Sham-Operated Rats after Various In Vivo Labeling Schedules

Exp. No. and treatment	Labeling period	Results 2–3 days postlabel		Results 3–4 wk postlabel	
		Per cent labeled small lymphocytes	Median grain count	Per cent labeled small lymphocytes	Median grain count
	(*days*)				
Sham					
6	1	12*	14‡	3*	7‡
		(6–18)	(4–>50)	(2–6)	(4–16)
8	4	11	24	7	9
		(8–14)	(5–>50)	(5–10)	(4–>50)
7	10	16	36	8	17
		(9–27)	(9–>50)	(7–9)	(4–>50)
Thymectomy					
8	4	24	20	—	—
		(19–28)	(5–>50)		
7	10	23	32	8	14
		(11–30)	(11–>50)	(6–10)	(4–>50)

* Mean per cent (and range) labeled small lymphocytes in blood from three to six rats, 200 or more lymphocytes scored on smears from each rat.

‡ Median (and range) grain count of labeled small lymphocytes.

thymectomy, the maximum number of labeled lymphocytes in the blood soon after labeling was always higher than in sham-operated controls and frequently averaged twice the control levels. By 3–5 wk after labeling, however, the frequency of labeled lymphocytes in the blood was the same in both groups. It was also apparent that adult thymectomy had no demonstrable effect on grain counts of labeled lymphocytes, either in the early or the late postlabeling periods (Tables I and III).

Combined Thymectomy and Sublethal Irradiation.—The effect of combined thymectomy and sublethal irradiation on the frequency of labeled peripheral blood lymphocytes is shown in Fig. 3 and Table III. These animals had been thymectomized or sham-operated 54 days before the start of the labeling period

and were exposed to 600 R whole body X-irradiation (300 R to a side) 7 days after operation.

During the 3 wk these animals were studied after 4 days of ^{3}H-TdR injections, no demonstrable effect of irradiation was apparent on the frequency of labeled lymphocytes in the peripheral blood. By contrast, thymectomy, with or without irradiation, consistently resulted in elevated levels of labeled

TABLE II

Labeling Profile of Mitoses (H-ARC) in the MLI with Cells from Thymectomized and Sham-Operated Rats after Various Labeling Schedules

Exp. No and treatment	Labeling period	Results of cultures established 0–3 days postlabel		Results of cultures established 3–5 wk postlabel	
		Per cent labeled mitoses	Median grain count	Per cent labeled mitoses	Median grain count
	(*days*)				
Sham					
6	1	10*	19‡	4*	12‡
		(6–17)	(7–>50)	(2–6)	(7–22)
8	4	90	27	5	21
		(77–96)	(7–>50)	(3–6)	(7–>50)
7	10	77§	24§	12	18
		(66–89)	(6–>50)	(8–17)	(6–>50)
Thymectomy					
8	4	87	25	—	—
		(75–95)	(7–>50)		
7	10	71§	23§	10	17
		(69–76)	(7–>50)	(9–12)	(7–>50)

* Mean per cent (and range) labeled mitoses in MLI; each value based on cultures from three to six rats; at least 100 mitoses scored/rat.

‡ Median (and range) grain count of labeled mitoses.

§ Cultured on 4th day of a 10 day labeling period.

lymphocytes in the blood during the 1st wk after labeling but apparently not thereafter (Fig. 3).

When the various groups of animals were compared with respect to the median grain count of labeled peripheral blood lymphocytes, a more rapid decline in grain count in irradiated animals was noticed. Furthermore, no heavily labeled cells (>30 grains) remained in the circulation in the late post-labeling period in the irradiated groups.

Taken together, the thymectomy and irradiation data clearly indicate that long-lived circulating lymphocytes in the rat can result from proliferation occurring in sites other than the thymus, and further that the precursors of such long-lived cells are not eradicated by an X-ray dose of 600 R.

Labeled Mitoses in the Mixed Lymphocyte Interaction (MLI).—Peripheral blood lymphocytes were obtained from ^{3}H-TdR–injected rats during both the early and late postlabeling periods and were stimulated with lymphocytes from F_1 hybrid donors bearing homologous H isoantigens. Colchicine was included in the cultures from the outset so that the mitotic figures scored included only

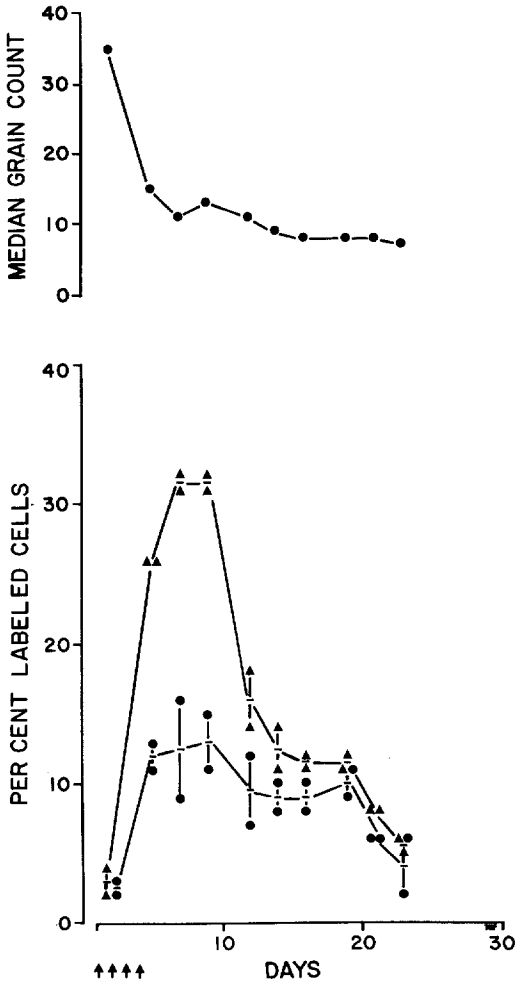

FIG. 3. Labeling profile of peripheral blood small lymphocytes in sublethally irradiated rats (600 R), with and without thymectomy. *Upper Graph*: Grain count data on peripheral blood small lymphocytes (each point represents the mean of median grain counts from four rats: two thymectomized, two sham-operated). *Lower Graph*: Per cent labeled lymphocytes in blood (▲—▲) = thymectomized, (●—●) = sham-operated. Each point = one rat; (—) = mean of values; (↑) = days of ^{3}H-TdR injections.

lymphocytes dividing for the first time in culture. The frequency of labeled mitoses in mixed cultures initiated at various times after in vivo labeling are indicated in Table II and also in Figs. 1 and 2. The length of the culture period did not have a significant effect on the frequency of labeled mitoses, and so data from different days of culture are combined.

In the cultures from intact animals initiated during or shortly after in vivo labeling for several days, 65–95% of the mitoses observed in the cultures were labeled (Table II), even though at this time only about 10% of the peripheral blood lymphocytes used to establish the cultures were labeled (Table I). In

TABLE III

The Effect of Adult Thymectomy Combined with Sublethal X-Irradiation on the MLI and on the Labeling Profile of Lymphocytes in the Peripheral Blood

Exp. No.	Treatment	Labeling period	Results 2 days postlabel		Results 18-20 days postlabel		MLI ^{3}H-TdR‖ incorporation day 5
			Per cent labeled small lymphocytes	Median grain count	Per cent labeled small lymphocytes	Median grain count	
		(*days*)					(*cpm*)
10	T_x* + X-irrad.	4	26‡ (24–27)	15§ (6–50)	7‡ (4–12)	7§ (5–23)	79, 68, 71, 180
10	Sham + X-irrad.	4	12 (11–13)	15 (6–>50)	5 (1–9)	8 (5–29)	971, 1233, 1104, 1480
8	T_x	4	24 (19–28)	20 (5–>50)	—	—	734, 673, 804, 1214
8	Sham	4	11 (8–14)	24 (5–>50)	7 (5–10)	9 (4–>50)	1843, 1240, 1883, 1524

* T_x = thymectomy.

‡ Mean per cent (and range) labeled small lymphocytes in blood from two to four rats, 200 or more lymphocytes scored on smears from each rat.

§ Median (and range) grain count of labeled small lymphocytes.

‖ These data obtained from other experiments involving comparably treated animals, values represent means of triplicate cultures established from each of four DA strain animals stimulated for 5 days with DA/BNF$_1$ cells.

cultures set up at later times (3–5 wk postlabeling), on the other hand, the percentage of labeled mitoses was approximately equal to that of labeled lymphocytes in the peripheral blood at that time. Therefore, it is apparent that lymphocytes which comprise the H-ARC pool and which proliferate in the MLI are predominately, but not exclusively, cells which have recently divided in vivo. This further implies that most of the labeled lymphocytes scored as long-lived in the peripheral blood are not H-ARC.

Additional information concerning this latter point was obtained in several experiments comparing the frequency of labeled lymphocytes in the blood and labeled mitoses in the MLI at much longer periods (approximately 200 days) after 2–10 days of labeling in vivo. These are summarized in Table IV. No

labeled mitoses were identified in the MLI initiated 200 days after in vivo labeling, although a few labeled lymphocytes were still present in the peripheral blood including some with more than six grains. Although fragmentary, these data again suggest that while the peripheral blood of the rat does contain a few very long-lived small lymphocytes, these are unlikely to be H-ARC.

Grain count data on labeled mitoses in mixed reactions established at various times after labeling are also included in Table II. These data show: (*a*) In cultures set up both in the early and late postlabeling periods, the grain count over mitotic figures after several days of injections was consistently higher than after labeling for only 1 day in vivo. (*b*) Unlike the median grain counts of peripheral blood lymphocytes (Table I), grain counts over the mitoses in the MLI did not decline precipitously with time in the postlabeling periods. Table II shows that the grain counts over H-ARC mitoses 3–5 wk after labeling were

TABLE IV

Frequency of Labeled Lymphocytes in the Peripheral Blood and of Labeled Mitoses in the MLI (H-ARC) at Long Periods after Labeling

Exp. No.	Days postlabel	Per cent labeled small lymphocytes (blood)	Per cent labeled mitoses in MLI (H-ARC)
5	62	3 (16/600)	1 (3/463)
4	199	<1 (2/600)	0 (0/86)
3	202	1 (9/900)	0 (0/202)

only moderately reduced (approximately 30%) from the levels observed in the early cultures; moreover, in other experiments, the decrease with time was even less. (*c*) Particularly with animals labeled for 1 or 4 days, the median grain count of responsive H-ARC (mitotic figures) in cultures established several wk after labeling was consistently higher than the median grain count of the lymphocyte population from which the cultures were established.

Thus, although some long-lived lymphocytes in the peripheral blood appear to undergo intermittent single divisions resulting in a lower median grain count with time, these apparently do not include the majority of H-ARC which display a relatively stable grain count level for several wk after labeling. The frequency of labeled H-ARC mitotic figures, however, does drop with time at a rate which may be faster than that for long-lived lymphocytes in general.

These findings suggest that long-lived H-ARC are less likely to undergo occasional divisions than the general long-lived lymphocyte pool, but rather that they disappear suddenly from the long-lived pool by death or by some process in which the grain count is rapidly diluted below demonstrable levels,

such as sequential multiple divisions. This disappearance from the labeled pool is apparently random, since the median grain count of the H-ARC population changes only slightly with time, i.e., there is no selection for or against heavily labeled cells.

Adult Thymectomy.—The effect of adult thymectomy on the frequency of labeled mitoses in the mixed lymphocyte interaction is indicated in Fig. 2 and Table II. Although the total proliferative response in the MLI gradually falls off after adult thymectomy (6), and in these experiments 8 wk post-thymectomy was at approximately ⅔ of normal (see Table III), it is clear that the predominance of newly formed cells in the MLI was characteristic not only of intact animals but of thymectomized ones as well. Furthermore, adult thymectomy did not eliminate, or even significantly reduce, the proportion of long-lived lymphocytes proliferating in cultures initiated 3–5 wk after labeling in vivo.

It was not possible to obtain any data on the age of lymphocytes proliferating in mixed reactions from animals subjected to combined sublethal irradiation and adult thymectomy, as this treatment effectively eliminated the proliferative response of cells from these animals in the MLI. This fact is apparent from Table III where the effect of thymectomy and irradiation, both singly and combined, on the total proliferative response in the MLI is illustrated.

Possible Influence of Age.—Although the general picture of predominance of young cells in the MLI was observed in four of the five experiments with multi-day labeling included in this report, one experiment with older animals was done and produced contrasting results. Six animals 16 wk of age were labeled and compared with rats 8–9 wk old in two other experiments. The results are summarized in Table V.

Although the percentage of labeled lymphocytes in the peripheral blood was similar in all three groups shortly after labeling, the percentage of labeled mitoses in mixed cultures set up at the same time ranged only from 2–30% (mean = 10%) among the six older animals as compared to the values of 66–95% (mean = 77, 90%) in the two younger groups. The grain count data on the three groups did not reveal notable differences.

At later times, the number of labeled lymphocytes remaining in the peripheral blood of the older animals was unusually low and very few were heavily labeled, but this group was not restudied until 6 wk after labeling as opposed to 3 or 4 wk for the other two groups.

It is clear that in the older group, relatively few H-ARC, and perhaps few long-lived cells in general, were among the lymphocytes being produced at the time of in vivo labeling. From these data it is tempting to speculate that this deficiency, as compared to the apparently high continuous output of H-ARC in the younger rats, is an effect of age, but more experiments are needed. The discrepancy is not simply related to general immunological deficiency in this

particular group of older rats, since in companion experiments involving injection with homologous cells, both this group and younger rats were able to produce large numbers of new H-ARC specifically responsive to the sensitizing antigen.[2]

TABLE V

Labeling Profile of Peripheral Blood Lymphocytes and of Mitoses (H-ARC) in MLI from Rats of Different Ages

Exp. No.	Age of rats at time of label	Labeling period	Results 0–2 days postlabel				Results 3–6 wk postlabel			
			Blood smears		MLI		Blood smears		MLI	
			Per cent labeled small lympho-cytes	Median grain count	Per cent labeled mitoses	Median grain count	Per cent labeled small lympho-cytes	Median grain count	Per cent labeled mitoses	Median grain count
	(weeks)	*(days)*								
8	9	4	11 (8–14)	24 (5–>50)	90 (77–96)	27 (7–>50)	7 (5–10)	9 (4–>50)	5 (3–6)	21 (7–>50)
7	8½	4	10* (5–14)	38 (7–>50)	77 (66–89)	24 (6–>50)	8‡ (7–9)	17 (4–>50)	12 (8–17)	18 (6–>50)
9	16	4	9 (6–11)	20 (4–>50)	10 (2–30)	29 (6–>50)	1 (1–2)	11 (4–25)	2 (0–4)	25 (8–>50)

Each value derived from panels of four to six rats.

* 4th day of 10 day labeling period.

‡ Animals labeled for 10 days.

DISCUSSION

The present findings allow some definite conclusions and prompt some interesting speculations about the large numbers of cells in the circulating lymphocyte pool of the rat which are responsive in the mixed lymphocyte interaction (H-ARC). Among the conclusions, may be listed the following: (*a*) In rats, some H-ARC have a long life-span in vivo without dividing, but the majority of the lymphocytes which proliferate in the MLI are the products of recent divisions in the body. As shown in Table II, for instance, injection of ^{3}H-TdR for several days labeled 65–95% of the cells dividing a few days later in the MLI. (*b*) Adult thymectomy does not eliminate the capacity of a rat to produce long-lived lymphocytes, some of which prove to be H-ARC. Thus, if one accepts the evidence that the cells responsive in the MLI are originally of thymic origin (6), it follows that they need not have recently emigrated from that site. Moreover, additional studies, still in progress, indicate that if an X-irradiated, thymectomized animal is restored to immunologic responsiveness with thoracic duct lymphocytes, nearly all of his H-ARC (as observed in the

[2] Wilson, D. B., and P. C. Nowell. Studies on the life history of lymphocytes. II. Generation during immunization of long-lived cells reactive in the mixed lymphocyte interaction and a consideration of their role in immunologic memory. In preparation.

MLI) for at least 2 months thereafter are of donor origin. Apparently H-ARC can be produced from existing thymus-derived cells already in the circulating pool. (*c*) Sublethal irradiation combined with adult thymectomy also does not eliminate the capacity of a rat to produce some long-lived lymphocytes which appear in the peripheral blood, but the inability of cells from these animals to produce a demonstrable MLI indicates that few if any of these are H-ARC. The data presented in Table III indicate these effects of thymectomy and of irradiation. (*d*) H-ARC and other long-lived cells as well appear to go through a series of rapid multiple divisions before their entrance into the peripheral blood. Thereafter, occasional single divisions may occur until death, sequestration, or another series of multiple divisions removes them from the circulating pool. H-ARC seem less likely to undergo occasional single divisions during their life-span than other types of circulating long-lived lymphocytes.

The data in Tables I and II show that injection of radioisotope for 4 or 10 days did not increase the number of labeled lymphocytes in the blood 4-fold or 10-fold as compared to injection for 1 day, but did produce striking increases in the median grain count of both labeled lymphocytes and mitoses. This strongly suggests that the production of circulating H-ARC and other long-lived lymphocytes involves multiple divisions extending over several days. The possibility of occasional single divisions in the long-lived lymphocyte pool is supported by the decrease in median grain count with time in Tables I and II, but this was less marked among H-ARC than in the lymphocyte population as a whole. The rapid decrease with time of per cent labeled mitoses in the MLI with relatively little decrease in median grain count (Table II) is consistent with the view that if an H-ARC divides at all, it usually goes through multiple divisions, diluting out the isotope, and so is no longer recognizable as a labeled cell.

Taken together, these four conclusions and the previous information on the large size and relative constancy of the H-ARC pool (3, 5) permit a speculative model to be developed on the life history of the H-ARC which can be tested against known facts and which can also suggest directions for future work. Drawing upon the investigations of others as well as our own studies, the model can include consideration of the entire long-lived lymphocyte pool, the H-ARC pool as a subdivision of it, quantitative relationships between different types of circulating lymphocytes, and permutations in these relationships.

The Circulating Long-Lived Lymphocyte Pool.—The peripheral blood of the rat contains lymphocytes of both thymic and bone marrow origin. Some can survive for long periods without dividing or with only occasional single divisions. Most evidence indicates that these long-lived cells in the blood are of thymic origin, and they would include H-ARC as well as other immunocompetent lymphocytes active in delayed hypersensitivity reactions or involved as "helpers" in antibody production (8, 9). Our present data would indicate

that although originally derived from the thymus, these long-lived cells can proliferate at extrathymic sites, when triggered by antigen or other mechanisms, and this proliferation characteristically involves multiple rapid divisions.

Life History of the H-ARC.—In this speculative model of long-lived lymphocytes in the rat, the cells responsive in the MLI (H-ARC) are a large subgroup with many of the characteristics of the entire population. H-ARC are produced in the thymus and then enter the circulating pool in small numbers. From time to time during their traffic between the blood and lymph, cells from this pool are triggered into a proliferative cycle involving a number of rapid sequential divisions. Proliferation would, of course, be outside of the circulation and presumably would normally take place in "thymus-derived" areas of the spleen and lymph nodes. The relationship of this proliferative activity to the functional importance of H-ARC in the general economy of the animal is not yet understood, but it probably involves the ultimate release into the circulation of progeny to perpetuate the circulating pool of H-ARC, as well as the continual utilization of these cells in some homeostatic mechanisms, perhaps including "surveillance" against neoplasia or other altered cells (10).

During their existence as long-lived recirculating cells, H-ARC undergo intermittent single divisions only rarely, but when properly triggered, either by some internally derived stimulus or external antigen, then would enter another phase of rapid multiple divisions. The interval between periods of rapid division for any given cell would be variable, depending upon chance exposure to the trigger mechanism, and one might also expect to find different time relationships in different animals depending on conditions. More data are needed under diverse circumstances, but the present experiments would suggest that for most H-ARC in the intact young rat, the interval between multiple division cycles would be a matter of a few days rather than several wk.

The total size of the circulating pool of lymphocytes responsive to major histocompatibility differences (H-ARC) would remain relatively constant. In the spleen and lymph nodes, cells would be removed from the circulating pool and produced for it at almost equal rates, with the slight deficit continually replaced by a small input of new circulating-pool cells from the thymus.

The triggering mechanism for rapid cell divisions among the H-ARC is uncertain except in those circumstances in which they are known to be exposed to a major histocompatibility difference e.g., the MLI in vitro and injection of AgB-disparate cells in vivo. It could reflect either an immunological surveillance function or some type of nonspecific stimulation.

There are some important distinctions between lymphocytes of the long-lived population, in general, and H-ARC, despite their common origin from the thymus and their common location in the circulating pool. First, on a cell-for-cell basis, the H-ARC are younger cells, having divided more recently in the body than has the average long-lived lymphocyte. Among very old lympho-

cytes (50–200 days), H-ARC are extremely rare. Secondly, those H-ARC which do survive for long periods in the circulating pool rarely undergo sporadic single divisions, while this appears to be a relatively common occurrence among other cells of the long-lived lymphocyte population. Finally, within the limits of the assay systems employed, combined thymectomy and sublethal irradiation appear to totally abrogate the production of H-ARC, although other long-lived lymphocytes are still being formed, perhaps from a few thymus-derived cells which survived the X-irradiation.

Quantitative Relationships among Different Types of Circulating Lymphocytes.—Based on present information (3, 11, 12), it is estimated that perhaps 50% of small lymphocytes in the peripheral blood of the rat at any one time would be H-ARC (2–4% directed against each of 15–20 AgB specificities). Another 25% might represent other long-lived thymus-derived cells, with the remaining 25% being lymphocytes of bone marrow origin. Obviously these proportions could be changed significantly by physiological and pathological permutations in the animal as well as by experimental conditions imposed upon it.

Permutations in the Circulating Lymphocyte Pool.—Experimental manipulations such as thymectomy and irradiation would be expected to produce demonstrable alterations in the relative proportions of H-ARC and other lymphocytes in the circulation. For instance, the consistently increased frequency of labeled lymphocytes immediately after ^{3}H-TdR labeling in adult thymectomized animals (Table III) may reflect decreased production of long-lived cells and, hence, an increased fraction of short-lived marrow-derived cells among those in the circulating pool. Also combined thymectomy and sublethal irradiation apparently eliminated H-ARC production completely, although other long-lived cells were still being formed.

As another experimental permutation, active sensitization of rats by cells differing at the AgB locus would be expected to result in proliferation in vivo of specifically responsive H-ARC from the circulating pool and the prompt appearance of many newly formed progeny. Mixed cultures initiated at this time against the sensitizing cells should consist very largely of recently divided host cells, whether or not the host was in a generally active state of production of H-ARC. This hypothesis is currently being tested.

Finally, the suggestion in the present work that older rats may be routinely less active than younger animals in the day-to-day production of newly formed H-ARC could, if confirmed by further studies, represent a permutation in the long-lived lymphocyte pool resulting not from experimentally imposed conditions but from the aging process itself.

These various theoretical considerations, and particularly the concept of the H-ARC as being potentially a long-lived cell, but one which in most animals is being frequently triggered into cycles of multiple divisions, do not appear to be

inconsistent with related observations by other workers. Data based on radiation-induced chromosome damage have indicated that relatively few circulating lymphocytes responsive in the MLI or to phytohemagglutinin (PHA) survive for long periods in the rat without division, in contrast to the findings in man where such cells are common (13, 14). Furthermore, Clancy and Rieke (15) reported that rat thoracic duct cells proliferating in mixed lymphocyte reactions or in response to PHA appeared to be largely cells which had recently divided in vivo. Tyler, Ginsberg, and Everett (16) showed that the proliferative and cytotoxic response of rat lymphocytes to mouse cells in vitro could involve a proportion of long-lived cells, but they did not rule out the possibility that most of the responsive cells had divided recently in the body. Similarly, the work of Gowans, with primary graft-*versus*-host reactions (17), and of Gowans and Uhr, on secondary responses to bacteriophage (18) which identified the small lymphocyte from the thoracic duct as the responding cell in both instances, did not indicate how recently these cells had previously divided in vivo. Clearly, more information is needed on the various components of the thymus-derived lymphocyte pool and on the stimuli, antigenic or otherwise, which determine the frequency of cell division of these various cell types.

SUMMARY

The life history, within the rat, of lymphocytes responsive to histocompatibility isoantigens in the mixed lymphocyte interaction was examined by the use of in vivo labeling with tritiated thymidine and radioautography. Lymphocytes in the peripheral blood and H-ARC (mitotic figures in the MLI) were compared with respect to the frequency of labeled cells and the median grain count. The following conclusions were drawn from this study: (*a*) Although some can be considered long-lived, the majority of H-ARC are the products of recent divisions in the body. (*b*) Adult thymectomy does not eliminate the production of long-lived lymphocytes, some of which are H-ARC. Hence, in addition to direct origin in the thymus, H-ARC, as well as other lymphocytes of the long-lived lymphocyte population, may derive from already existing thymus-derived cells in the circulation and thymus-dependent areas of the secondary lymphoid tissues. (*c*) Sublethal X-irradiation (600 R) in combination with adult thymectomy does not eliminate the capacity to produce some long-lived lymphocytes, however, few if any are H-ARC. (*d*) H-ARC and other long-lived lymphocytes appear to go through a series of rapid multiple divisions before they enter the circulation. Thereafter, long-lived lymphocytes appear to undergo intermittent single divisions which decrease both the frequency and median grain count of labeled cells gradually with time. On the other hand, labeled H-ARC maintain a more stable grain count despite a rapid decrease in frequency with time. This is taken to indicate that H-ARC are less likely to undergo occasional single divisions during their life-span, but may undergo periodic rapid sequential divisions.

A speculative model is developed from these data on the life history of H-ARC which may be of predictive value in future studies and which can be tested against known facts.

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REFERENCES

1. Wilson, D. B. 1967. Quantitative studies on the mixed lymphocyte interaction in rats. I. Conditions and parameters of response. *J. Exp. Med.* **126**:625.
2. Wilson, D. B., W. K. Silvers, and P. C. Nowell. 1967. Quantitative studies on the mixed lymphocyte interaction in rats. II. Relationships of the proliferative response to immunologic status of the donors. *J. Exp. Med.* **126**:655.
3. Wilson, D. B., J. L. Blyth, and P. C. Nowell. 1968. Quantitative studies on the mixed lymphocyte interaction in rats. III. Kinetics of the response. *J. Exp. Med.* **128**:1157.
4. Wilson, D. B., and P. C. Nowell. 1970. Quantitative studies on the mixed lymphocyte interaction in rats. IV. Immunologic potentiality of the responding cells. *J. Exp. Med.* **131**:391.
5. Wilson, D. B., and P. C. Nowell. 1971. Quantitative studies on the mixed lymphocyte interaction in rats. V. Tempo and specificity of the proliferative response and the number of reactive cells from immunized donors. *J. Exp. Med.* **133**:442.
6. Johnston, J. M., D. B. Wilson, and P. C. Nowell. Origin of Immuno-Reactive Lymphoid Cells in the Rat. Proceedings Fourth Leukocyte Culture Conference. Appleton-Century-Crofts Inc., New York. In press.
7. Moorhead, P. S., P. C. Nowell, W. J. Mellman, D. M. Battips, and D. A. Hungerford. 1960. Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp. Cell Res.* **20**:613.
8. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen-reactive cells. *Transplant. Rev.* **1**:3.
9. Miller, J. F. A. P., and D. Osoba. 1967. Current concepts of the immunological function of the thymus. *Physiol. Rev.* **47**:437.
10. Brook Lodge Symposium on Immunological Surveillance. 1970. R. T. Smith and M. Landy, editors. Academic Press Inc., New York.
11. Simonsen, M. 1967. The clonal selection hypothesis evaluated by grafted cells reacting against their hosts. *Cold Spring Harbor Symp. Quant. Biol.* **32**:517.
12. Simmons, M. J., and R. Fowler. 1966. Chorioallantoic membrane lesions produced by inoculation of adult fowl small lymphocytes. *Nature (London).* **209**:588.
13. Human Radiation Cytogenetics. 1967. H. J. Evans, W. M. Court Brown, and A. S. McLean, editors. North Holland Publishing Co., Amsterdam, The Netherlands.
14. Nowell, P. C., B. E. Hirsch, D. H. Fox, and D. B. Wilson. 1970. Evidence for the existence of multipotential lympho-hematopoietic stem cells in the adult rat. *J. Cell. Physiol.* **75**:151.

15. Clancy, J., and W. O. Rieke. 1969. The *In Vitro* Response of Short- and Long-Lived Small Lymphocytes to F_1 Hybrid Cells. W. O. Rieke, editor. Proceedings Third Annual Leukocyte Culture Conference. Appleton-Century-Crofts Inc., New York. 465.
16. Tyler, R. W., H. Ginsburg, and N. B. Everett. 1969. The Response of Rat Thoracic Duct Lymphocytes Cultured on Mouse Monolayers. W. O. Rieke, editor. Proceedings Third Annual Leukocyte Culture Conference. Appleton-Century-Crofts Inc., New York. 451.
17. Gowans, J. L., and D. D. McGregor. 1965. The immunological activities of lymphocytes. *Progr. Allergy.* **9**:1.
18. Gowans, J. L., and J. W. Uhr. 1966. The carriage of immunological memory by small lymphocytes in the rat. *J. Exp. Med.* **124**:1017.