PROLIFERATION IN VIVO OF T LYMPHOCYTES REACTIVE TO HISTOCOMPATIBILITY ALLOANTIGENS: A CORRECTION*

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In recent reports on the biological properties of lymphocytes reactive to histocompatibility antigens (HARC) (1-3), we have presented evidence in support of several generalizations. These include: (a) That in the peripheral blood and thoracic duct lymphocyte population, the frequency of HARC reactive to any strong H alloantigen is high, on the order of several percent of the total recirculating lymphocyte population (4). (b) That the majority of HARC in the blood (50-90%) are recently divided (1), suggesting frequent turnover in vivo. (c) That these young cells in the blood are largely excluded from recirculation in the lymph (3). (d) That immunization across strong allogeneic differences increases the proportion of recently divided HARC among the specifically responsive population in the circulation and results in an accelerated proliferative response in the mixed lymphocyte interaction (MLI) (5).

As studies on the life history of HARC have proceeded in our laboratory and elsewhere, an apparent paradox has been increasingly difficult to explain. The total number of HARC reactive to the many haplotypes in the species does appear to be very large, and may indeed involve the entire T-cell population (2, 6-8), but studies by various methods indicate that a significant proportion of circulating T cells are long-lived and not recently divided (9-11). Thus, the conclusion that most HARC are young cells seemed to require reexamination, including the possibility of experimental error.

Our view concerning the frequency of newly formed HARC was largely based on enumeration of labeled mitoses in colchicine-blocked mixed lymphocyte cultures which were derived from rats labeled in vivo with tritiated thymidine ([3H]TdR). The proportion of labeled mitoses was consistently high in experiments involving blood from recently labeled animals, and low when the blood was obtained several weeks after labeling or when thoracic duct lymph was used. Although the reutilization of [3H]TdR was considered as an explanation for the high values, the low results in the other cultures, as well as grain count data and negative experiments with labeled supernates, caused us to discount this possibility (3). Therefore, the use of cold thymidine in the cultures (10) was considered unnecessary and was avoided because of possible inhibitory effects on proliferation. However, the realization that high frequencies of labeled mitoses occurred only in peripheral blood cultures established soon after labeling, in which there was some contamination with labeled granulocytes, led to further investigation of

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this possible source of error and subsequent reevaluation of our conclusions concerning the life history of HARC.

Materials and Methods

The techniques for in vivo labeling of lymphocytes and enumeration of labeled mitoses in the MLI were similar to those previously described (1, 3). Briefly, rats of the inbred Lewis strain were injected intraperitoneally with [3H]Tdr once or twice daily for 4 days (0.7-1.0 μCi/g body weight per injection; spec act = 6.7 μCi/mmol). Mixed lymphocyte cultures were established with peripheral blood lymphocytes (PBL) or thoracic duct lymphocytes (TDL) from labeled parental donors and unlabeled F1 donors 2 days or 23 days after the end of the labeling period. The peripheral blood lymphocyte suspension was obtained by dextran sedimentation and was normally contaminated by 10–20% granulocytes. The cultures were terminated 2–4 days after initiation and the percentage of labeled mitoses determined by radioautography. Studies were scored “blind” by two observers and the results pooled. All cultures contained colchicine throughout the culture period, so that only “first division” mitoses were observed.

Results

Reutilization of Label from Granulocytes. In order to test the effect of cold thymidine on labeling patterns in the MLI, parallel peripheral blood lymphocyte cultures were set up from six rats 2 days after the end of the in vivo labeling period, with and without the addition of cold thymidine (12.5 μg/ml). The results are shown in Table I.

The frequency of labeled mitoses in the cultures without cold thymidine averaged 62%, a result very similar to our previous studies (1-3). In the cultures with cold thymidine, only 6% of the mitoses were labeled, although the total mitotic activity in the two groups was similar. Blood smears obtained from the donor rats when the cultures were established showed an average of 11% of the circulating small lymphocytes to be labeled. These data, and similar findings in several subsequent experiments, strongly suggested that most cells responsive in the MLI are not newly formed cells in vivo and that reutilization of label in vitro in the absence of cold thymidine had earlier led to erroneous conclusions.

To test directly the possibility that contaminating granulocytes could be a source of [3H]Tdr for reutilization by lymphocytes in the MLI, additional experiments were done. Cultures with and without cold thymidine were established in which the parental cells were approximately a 6:1 mixture of thoracic duct lymphocytes from an unlabeled rat and purified granulocytes from a labeled rat of the same strain. The granulocytes (92% pure) were obtained from peripheral blood by gradient centrifugation twice through Ficoll-Hypaque (12).

| Table I |
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| **Effect of Cold Thymidine on the Frequency of Labeled Mitoses in PBL Mixed Lymphocyte Cultures from Recently Labeled Rats** |
| No. labeled mitoses* | Labeled mitoses | Mitotic index (mit./1,000 cells) |
| PBL MLI with cold thymidine | 28/525 | 6 | 3.0 |
| PBL MLI without cold thymidine | 301/476 | 62 | 3.3 |

* Parallel cultures established from six labeled rats.
The results, summarized in Table II, strikingly demonstrate the ability of labeled granulocytes to provide \[^{3}H\]TdR to proliferating lymphocytes, and the complete prevention of this phenomenon by cold thymidine. In two experiments, an average of 24\% of the lymphocyte mitoses were labeled in the absence of cold thymidine; none were labeled when it was present.

**Effect of Immunization; Recirculation.** Two further experiments with cold thymidine were done to determine if modifications were necessary in our previous conclusions concerning the effect of immunization on the frequency of newly formed HARC and the ability of such cells to recirculate in the lymph.

Lewis rats were immunized intraperitoneally as previously described (5) with allogeneic (DA) spleen cells. 7 days later (2 days after the end of the labeling period) both PBL and TDL mixed lymphocyte cultures were established against \(F_1\) cells bearing the specific immunizing alloantigen (L/DA) and against \(F_1\) cells bearing a nonspecific alloantigen (L/BN). Similar cultures from nonimmunized hosts were established as controls. The entire experiment was repeated 23 days after the end of the labeling period. All cultures contained cold thymidine (12.5 \(\mu\text{g/ml}\)).

**TABLE II**

**Effect of Cold Thymidine and Added Labeled Granulocytes on the Frequency of Labeled Mitoses in TDL Mixed Lymphocyte Cultures from Unlabeled Rats**

| No. labeled mitoses | Labeled mitoses |
|---------------------|-----------------|
| TDL MLI with cold thymidine | 0/176 | 0 |
| TDL MLI without cold thymidine | 54/225 | 24 |

*Sum of two experiments. Labeled granulocytes added to all cultures.

The results of one experiment are summarized in Table III, and similar results were obtained in a second experiment. They confirm our previous observation that the frequency of specifically responsive newly formed HARC in the blood can be significantly raised by immunization, but otherwise indicate that the frequency of both young and long-lived HARC in the blood and lymph is similar to that of the general circulating lymphocyte population in immunized and in non-immunized rats. The data are not adequate, however, to rule out definitely some exclusion from the lymph of cells recently formed in response to immunization.

**Discussion**

It seems that unrecognized reutilization of \[^{3}H\]TdR from contaminating labeled granulocytes led us previously to erroneous conclusions concerning the high frequency of labeled mitoses in peripheral blood mixed lymphocyte cultures derived from recently labeled rats. The problem did not occur in TDL cultures, or in PBL cultures established from rats sufficiently long after injection of \[^{3}H\]TdR so that labeled granulocytes were no longer present in the blood. Rieke (13) has demonstrated in vivo that reutilization can become significant when DNA-synthesizing cells are in close contact with disintegrating labeled cells, and the early death of most granulocytes in the MLI would provide these conditions. The
mechanism of reutilization is not known, but cell-to-cell proximity seems important, as we were earlier unable to demonstrate measurable reutilization when unlabeled lymphocytes in an actively proliferating MLI were exposed to cell-free supernates containing as many as 100,000 dpm’s of $[^3H]$TdR per ml.

Based on the results to date, there seems no reason to modify two of the generalizations stated earlier—the large number of HARC in the circulation appears now well established (2, 8), and the increased proportion of young HARC in the blood produced by immunization was confirmed in the present study. The following modifications do seem appropriate for the other two generalizations: (a) The age distribution of circulating HARC in nonimmunized rats does not appear to differ significantly from that of the total recirculating lymphocyte population in the blood and lymph, and, (b) at least in nonimmunized rats, newly formed HARC can apparently move into the lymph and recirculate as readily as long-lived cells. The possibility of some exclusion from recirculation of HARC recently stimulated to divide by specific immunization must be examined further, as our limited data are inconclusive, and McGregor et al. (14) have reported that rat lymphocytes recently formed in response to infection with Listeria fail to recirculate. In general, it is increasingly difficult to conceive of distinct “long-lived” and “short-lived” populations among recirculating lymphocytes. Instead, it is probably preferable to consider these cells as reflecting a continuous spectrum of time periods since their last division, with certain properties being more prominent toward either end of this continuum. The present findings argue against certain earlier speculations concerning the

### Table III

|                  | PBL MLI—        | PBL smear—      | TDL MLI—        | TDL smear—     |
|------------------|-----------------|-----------------|-----------------|---------------|
|                  | Lab. mitoses    | Lab. smear—     | Lab. mitoses    | Lab. smear—   |
|                  | Specific F$_1$  | Nonspecific F$_1$  | Specific F$_1$  | Nonspecific F$_1$  |
|                  | (L/DA)          | (L/BN)          | (L/DA)          | (L/BN)        |
| Early period—    |                 |                 |                 |               |
| cultures        |                 |                 |                 |               |
| established      |                 |                 |                 |               |
| 2 days post-label|                 |                 |                 |               |
| Lewis rats       |                 |                 |                 |               |
| immunized with   |                 |                 |                 |               |
| DA cells         |                 |                 |                 |               |
| Nonimmunized     |                 |                 |                 |               |
| Lewis rats       |                 |                 |                 |               |
| %                | %               | %               | %               | %             |
| Lewis rats       |                 |                 |                 |               |
| immunized with   |                 |                 |                 |               |
| DA cells         |                 |                 |                 |               |
| Nonimmunized     |                 |                 |                 |               |
| Lewis rats       |                 |                 |                 |               |
| %                | %               | %               | %               | %             |

* Each value derived from panels of three-four rats; 100-300 mitoses. Cold thymidine in all cultures.
† Very poor proliferative response; 6/16 mitoses labeled.
continuous proliferation of HARC in vivo as part of an immune surveillance mechanism against neoplasia, and still leave unanswered many basic questions about the biological importance of cellular responses to major histocompatibility alloantigens.

Summary
Rat lymphocytes in mixed cultures can reutilize tritiated thymidine from labeled granulocytes. Shortly after thymidine injections in vivo, major effects on the frequency of labeled lymphocyte mitoses in peripheral blood cultures are introduced by 10–20% polymorph contamination, even though transfer of label via supernates is not demonstrable.

Cold thymidine in the cultures prevents reutilization, and has permitted reevaluation of several previous conclusions concerning the life history of lymphocytes reactive to major histocompatibility alloantigens (HARC). Rather than being predominantly recently divided cells, HARC do not appear to have an age distribution, in blood or lymph, significantly different from the general recirculating lymphocyte population. However, the ability of immunization across strong allogeneic differences to increase markedly the proportion of young HARC among the specifically responsive population has been confirmed.

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