Brief Definitive Report

VERY RAPID DECAY OF MATURE B LYMPHOCYTES IN THE SPLEEN*

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Classical experiments by Osmond and Nossal (1) have demonstrated the continuous production of very high numbers of B lymphocytes in the bone marrow, but there are few indications that a significant fraction of these ever migrates to the periphery and participates in the establishment of available repertoires. "Half-lives" of lymphocytes have been determined by labeling or killing dividing cells, but these approaches do not resolve the question of whether the mitotic activity takes place among precursors that rapidly differentiate to competence and are exported to the periphery, or among mature, peripheral B lymphocytes. These two alternatives have very different consequences for the stability of available repertoires, because the expansion of mature B cells will result in the exclusion of novel clones produced in the marrow. This question is fundamental, because the size of the available antibody repertoire appears to be primarily limited by the numbers of different clones the individual contains at any given time. We have now devised an approach that provides the direct measurement of the persistence of mature B cells in the periphery, regardless of cell division. The results thus obtained indicate average decay rates of 50% per day for a large fraction of normal spleen B cells under quasi-physiologic conditions.

Materials and Methods

Mice. Male C57BL/6 (B6), C57BL/10. Sc.Sn (B10.Sn), and C57BL/10.Sc.Cr. (B10.Cr) were obtained from our own colony, from Olac, Blackthorn, England, or from Bomholtgaard, Ry, Denmark, respectively. They were used between 2 and 5 mo of age.

Cell Suspensions. Spleen cells were prepared as described (2), and separated by size at unit gravity in continuous gradients of bovine serum albumin (3). Lymphocyte fractions with a sedimentation velocity of <4 mm/h were pooled and used as small cells.

Cultures and Limiting Dilution Analysis. These were done as previously described (2). In brief, limited numbers of spleen cells were distributed in 0.2-ml cultures containing 6×10^5 rat thymus filler cells in medium containing 5×10^{-5} M 2-mercaptoethanol and 10% fetal calf serum (FCS). For each determination of the frequency of reactive B cells, at least 6 different cell concentrations were set up, with 48 replicate identical cultures per point. Whenever indicated, the cultures contained optimal concentrations (50 μg/ml) of lipopolysaccharide (LPS) from *Salmonella abortus equi* (Difco Laboratories, St. Louis, Mo.). All cultures were assayed on day 5 for IgM-secreting plaque-forming cells (PFC), in the protein A-plaque assay (4). Cultures containing >10 PFC were scored as positive, and the results were shown to conform to the first-order term of Poisson's distribution. The frequencies of reactive cells shown were

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derived from semi-log plots, and indicate the numbers of cells per culture allowing 63% of the cultures to respond.

Cell Transfers. The small cell fraction purified from spleen cells was washed in balanced saline solution, adjusted to 50×10^6 cells/ml and injected intravenously into recipient mice.

Splenic Localization of Transferred Cells. Spleen cells were labeled with ^{51}Cr after removal of erythrocytes and dead cells as described in detail (5). They were then transferred intravenously into normal mice and, 24 h later the recipients were killed and the amount of radioactivity recovered in the spleen was measured and expressed as a percentage of the total cellular radioactivity injected.

Results and Discussion

The mouse strain C57BL/10Sc.Cr (B10.Cr) was previously found to carry a mutant gene at the *lps* locus in chromosome 4 (6) controlling the expression of a B cell receptor for LPS from gram-negative bacteria, which can be serologically (7) and functionally (2) identified in roughly one-third of all splenic B cells. B lymphocytes of this strain fail to recognize LPS as a polyclonal mitogen. The mutation must have occurred after this substrain was separated from the related C57BL/10Sc.Sn (B10.Sn) strain that shows full reactivity to this mitogen (8). No other differences between these strains are known, and they are fully histocompatible. All attempts to induce mutual serological or cellular immunity by grafting of cells or tissues in various immunization protocols have failed thus far (A. Coutinho, unpublished results). The same applies to another related strain, C57BL/6.

The strategy in these experiments was to follow the decay of LPS-responder B lymphocytes in histocompatible, untreated LPS-nonresponder recipients, using techniques that provide the direct count, by limiting dilution analysis, of all mitogen-reactive cells in a population (2). Because the spleen is the major site of migration for B lymphocytes newly formed in the marrow (1), we were concerned, in the present observations, with the turnover of splenic B cells. To minimize extraneous effects caused by differential homing patterns of lymphocyte populations, we have followed LPS-responder spleen cells in the recipient's spleen. Table I shows the limits of the method, namely the frequencies of splenic B cells from B10.Cr and B10.Sn that can be activated by LPS to grow as clones of IgM-secreting PFC, as well as those measured in the absence of LPS. The latter are probably stimulated by FCS components, and their frequencies are comparable in both strains of mice and to that obtained in B10.Cr mice in the presence of LPS (1:6,000 spleen cells), whereas in LPS-responders, 1:5 spleen cells is detected as an LPS-reactive clonal precursor. As also shown in Table I, in vitro mixtures of LPS-responder and nonresponder spleen cells, when analyzed for the frequency of LPS-reactive clones, provide the correct count; that is, the frequency expected from the independent measurements in each cell population.

To avoid stem-cell contamination in the spleen cell populations to be transferred, small lymphocytes were purified (3) and used in the transfers. It is well known that viable lymphocytes, after intravenous transfer, follow specific patterns of traffic and homing (5). By 24 h, the distribution of injected lymphocytes is thought to have stabilized and we therefore took this time as the starting point in our experiments. Two independent measurements of the numbers of transferred cells homing to the spleen were taken at 24 h. One involved the conventional technique of labeling the transferred cells with radioactive chromium and determining the fraction of the injected activity recovered in the recipient spleen. Confirming previous observations

TABLE I

Frequency of LPS-reactive Cells in the Spleen of LPS-responder and LPS-nonresponder Strains of Mice

	LPS	Without LPS
Experiment 1		
B10.Sn	1:5	1:5,500
B10.Cr	1:6,000	1:6,000
Experiment 2		
B6	1:12	ND‡
B6 + B10.Cr*		
expected	1:108	ND
observed	1:100	ND
B10.Cr	1:5,500	ND

* In mixing experiments, spleen cells from LPS-responder mice were diluted 1:9 with cells from LPS-nonresponder mice.

‡ Not determined.

(5), the results shown in Table II demonstrate that ~20% of the injected cells are present in the spleen 24 h after transfer. Radioactivity measurements at these early times reflect viable cell counts as shown by determining the numbers of LPS-reactive (donor) B cells present in the recipient spleen at the same time. Table II also shows that 20% of the injected cells can be recovered.

The removal of cells from the donor spleen and their purification and transfer certainly result in damage that influences the survival in the host. Therefore, the numbers of donor cells persisting in the host cannot be compared to the total number transferred. However, this difficulty is obviated if the numbers of donor cells found in the recipient spleen at 24 h are taken as the starting value in the experiment.

Thus, injured or dead cells do not circulate and are rapidly retained in nonlymphoid organs, primarily the liver (5). That is, the 24-h value represents the total numbers of cells that were successfully transferred and that will follow "normal" rates of decay in the host. This measure also corrects for the possibility that transferred cells home to organs other than spleen, as these are then excluded from the experiment. Frequency determinations of LPS-reactive cells in the recipient spleen were then performed in the days after transfer. These frequencies allowed for the calculation of the total numbers of LPS-reactive cells in the organ, and these were compared with the numbers found 24 h after transfer. Taking the fraction recovered at 24 h as the starting (100%) value, the logarithmic plot of the decay with time of LPS-reactive cells in the recipient spleen is shown in Fig. 1. These results were obtained in two independent experiments defining precisely the same curve, namely a decay rate of 50% per day. After day 5 of transfer, these methods become unsuitable, as the "background" frequencies in B10.Cr make it difficult or impossible to detect small numbers of cells.

The interpretation of these results must consider first the possibility that the rapid decay rate observed represents active elimination of donor cells in nonphysiologic conditions, rather than the normal dynamics in the system. This appears unlikely, however, as no histocompatibility reactions could be detected in this strain combination, even after very intense protocols of immunization. To directly assess this

TABLE II

*Number of Lymphocytes from LPS-responder Mice Recovered in the Spleen 24 h after Transfer into LPS-nonresponder Mice**

Frequency of LPS-reactive cells in recipient's spleen	Total number of LPS-reactive cells recovered in recipient's spleen	Percent of the transferred LPS-reactive cells in recipient's spleen‡	Percent of injected ^{51}Cr-labeled cells recovered in recipient's spleen
1:400	158,000	19.8	21.2

* All results are averages of three to five recipient mice.

‡ The frequency of LPS-reactive cells in the recipient's spleen was determined by limiting dilution analysis, and the number of LPS-reactive cells recovered is expressed as a percentage of the number of LPS-reactive cells injected, as assessed by limiting dilution analysis on the population of injected cells.

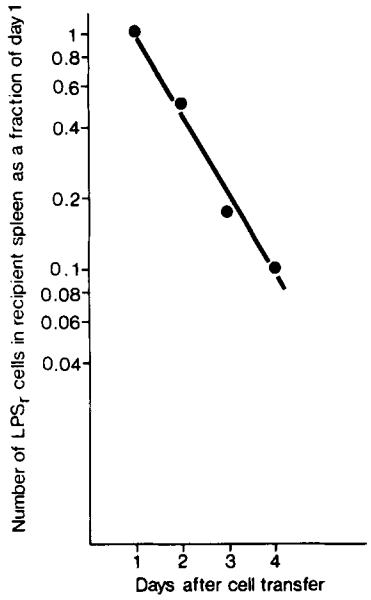

FIG. 1. Small spleen lymphocytes from LPS-responder mice were transferred intravenously into LPS-nonresponder mice. At the indicated times after transfer, recipients were killed and the frequencies of LPS-reactive B lymphocytes in spleen cells were determined. The total numbers of LPS-reactive cells remaining in the recipient spleen at the different time-points were then calculated as a fraction of the number of LPS-reactive cells found at 24 h after transfer. The figure shows the results obtained in two independent experiments.

possibility, identical experiments were performed using either normal recipients or B10.Cr mice that had been "immunized" 3 mo earlier with 10^7 B6 spleen cells, the donor inoculum used in these experiments. Results shown in Table III demonstrate that both the homing to the host spleen and the decay in the organ of LPS-reactive donor B cells are not affected by previous immunization of the recipients, making it very unlikely that the decay rates observed are the result of active elimination by immune mechanisms. Furthermore, the transfer of $<5 \times 10^6$ B cells represents a minor fraction of the total numbers produced daily in the normal system and therefore it should not be expected to introduce gross alterations in the physiologic turnover rates. The experimental approach described here provides conditions that are very little, if at all, immunogenic in contrast to previously used markers, e.g., allotypic. In addition,

TABLE III

Recovery of LPS-responder Lymphocytes from the Spleens of Normal or Immunized LPS-nonresponder Hosts

Day of assay	State of recipient	Frequency of LPS-reactive cells	Percent of transferred LPS-reactive cells in recipients spleen at 24 h	Donor cells persisting in spleen as a fraction of 24 h value
1	Normal	1:140	0.29	—
	Immune	1:125	0.31	—
4	Normal	1:1,400	—	0.1
	Immune	1:1,300	—	0.096

Normal B10.Cr mice, or mice given one intravenous injection of 10^7 spleen cells from B6 mice 3 mo previously, were used as recipients of B6 spleen cells, as described in the text. 1 and 4 d after transfer, spleen cells from groups of three mice were pooled and the frequencies of LPS-reactive cells were determined, as in Table I. Fractions of donor cells homing to the spleen at 24 h were determined as in Table II, and those of the persisting cells were determined as in Fig. 1.

our assay requires reactivity and functional performance of the transferred cells, thus excluding those still alive but already unreactive to competent stimuli that can be detected in other protocols. Finally, the responses are studied in vitro, and the functional performance is therefore not influenced by regulatory mechanisms or other conditions specific to the host environment.

It might be argued that the B cell population defined by reactivity to LPS is not a representative sample of the whole B cell compartment. It may well be that donor LPS-reactive cells in our experiments did not in fact decay in the recipient spleen, but simply differentiated to other stages along the pathways of B cell development with a concomitant loss of LPS-reactivity. Alternatively, LPS-reactive cells could have migrated out of the spleen to, for example, lymph nodes. In these cases, however, similar decay rates would apply for all stages in a given differentiative line or for the cells in the next lymphoid organ, as the B cell compartment is kept reasonably constant in the physiologic steady state.

It can be postulated, however, that LPS-reactive lymphocytes compose an independent subset that is distinct in rate of decay from the other two-thirds of splenic B cells. This possibility cannot be excluded, although it would appear unlikely, because we have previously found that LPS-reactive cells compare well with the rest of the B lymphocyte compartment in the rates of decay and recovery, after in vivo killing of cells in S-phase with hydroxyurea (A. A. Freitas, B. Rocha, L. Forni and A. Coutinho, manuscript submitted for publication).

The present results concern at least one-third of all splenic B cells. If they are taken as representative of the majority of B lymphocytes in the spleen, it would follow that $\sim 25 \times 10^6$ B cells would leave the organ (or die) in a day. These numbers are comparable to those determined by Osmond and Nassal (1) for the rate of B cell production in bone marrow, and these observations would therefore suggest that the majority of newly formed B cells migrate to the periphery and participate in the astonishingly high turnover rate described here. It should be pointed out, however, that although these very high decay rates could apply to the majority of splenic B cells, other B lymphocyte subsets might have considerably longer life spans. These differences may result from regulatory mechanisms operating at the periphery and selecting for long life a few members of the rapidly turning over output from the

marrow. This view might explain the wide discrepancies in the life span of B lymphocytes previously determined, which vary from 2 d to 7 wk, according to the experimental system and the clonal specificity analyzed (8–10).

Summary

To determine the persistence of immunocompetent B lymphocytes at the periphery, regardless of cell division, we have followed the decay of lipopolysaccharide (LPS)-reactive B cells in LPS-nonresponder, histocompatible hosts. Both the numbers of transferred cells and of those persisting in the recipients could be determined with precision by limiting dilution analysis of the various cell populations. Decay rates of 50% per day were determined. Because we could exclude immune elimination of donor cells and the numbers of transferred cells were too low to result in gross alterations of the physiologic turnover rates, we conclude that the majority of LPS-reactive B lymphocytes and a large part of the whole B cell compartment show this astonishingly high rate of decay.

The method introduced here might prove useful in a variety of clonal assays, as it can detect cells present at a very low frequency.

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