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Immunology

Natural effector T lymphocytes in normal mice

(helper T cells/suppressor T cells/"background" activity/normal immune system)

PABLO PEREIRA*, EVA-LOTTA LARSSON*, LUCIANA FORNI†, ANTÓNIO BANDEIRA*, AND ANTÓNIO COUTINHO*

*Laboratory of Immunobiology, Pasteur Institute, Paris, France; and †Basel Institute for Immunology, Basel, Switzerland

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ABSTRACT **The "natural" T-cell activity in normal unimmunized mice was studied. By double-parameter fluorescence-activated cell sorter analysis, it was found that 5–10% of all splenic Lyt-2$^+$ and L3T4$^+$ lymphocytes are large, of which more than half are in mitotic cycle. In contrast with small resting cells of the same phenotype, activated (large) T cells isolated from normal mice are functional effector cells: L3T4$^+$ large cells induce normal B lymphocytes into proliferation and antibody secretion, while large Lyt-2$^+$ cells efficiently suppress B-lymphocyte responses. No effector cell cytolytic activity could be detected among naturally activated T cells. The significance of these findings for the internal activity in the normal immune system is discussed.**

Much attention has been given in the past to natural antibodies, from both the theoretical (1) and the clinical points of view (2, 3). Astonishingly, the counterpart on the T-cell compartment of such natural activity of B lymphocytes has not been described thus far. The evaluation of current perspectives of an integrated immune system, which implies some degree of autonomous activity (4, 5), led us to investigate the presence of activated T cells in normal animals. The T-cell equivalents of "natural" plasma cells would be effector cells: helper (T_H), suppressor (T_S), or cytotoxic T lymphocytes (CTL). The availability of assay systems that selectively detect such effector functions regardless of clonal specificities (6, 7) makes it possible to investigate the presence of those cells. Furthermore, lymphocytes differentiate both in bone marrow and thymus to a resting state of small immunocompetent cells (8, 9), allowing the identification of activated cells in the periphery by their presence in the mitotic cycle. We report here that roughly 10% of all T lymphocytes in normal unimmunized mice are large blast cells, of which at least some exert effector functions—namely, helping or suppressing B lymphocytes.

MATERIALS AND METHODS

Mice. BALB/c mice, kept in conventional specific pathogen-free conditions, were used at ages between 8–12 weeks.

Antibodies and Reagents. The following monoclonal antibodies were used with rabbit complement in cytotoxic treatments: rat IgMκ, anti-Thy-1, J1J, a kind gift of J. Sprent (Scripps Clinic and Research Foundation, La Jolla, CA); mouse IgMκ, anti-Lyt-2.2, HO-2.2 (10); rat IgGκ, anti-L3T4, GK-1.5 (11); and mouse IgMκ, anti-I-$A^{b/d}$, B17-263 (12). These were used as ascitic fluids or culture supernatants at the appropriate dilutions. For immunofluorescence studies, the following biotin-conjugated monoclonal antibodies were used to identify the various lymphocyte classes: rat IgGκ, anti-μ chain, R33-60 (13); rat IgGκ, anti-Lyt-2, 53-6.72 (14); and rat IgGκ, anti-L3T4, H-129-19.69 (15). These were used after ion-exchange column purification from culture supernatants. Concanavalin A (Con A), Percoll, and protein A from *Staphylococcus aureus* were purchased from Pharmacia; *Salmonella abortus equi* lipopolysaccharide (LPS), from Difco; and α-methyl D-mannoside, from Sigma. Interleukin 2 (IL-2)-containing conditioned medium was prepared by Con A stimulation of rat spleen cells as described (16).

Immunofluorescence and Fluorescence-Activated Cell Sorter (FACS) Analysis. Splenic cell suspensions were stained for surface markers as described in detail (17) in a two-step procedure using biotin-labeled monoclonal antibodies to IgM, Lyt-2, and L3T4 (see above), followed by avidin-fluorescein conjugates. Cells were analyzed for forward light scatter and fluorescence intensity in a FACS II (Becton Dickinson). Staining for DNA content was performed with propidium iodide as described by Vindelov (18).

Cell Purifications. Large or small Lyt-2$^+$ or L3T4$^+$ lymphocytes were purified from normal spleen cells by two alternative procedures. In the first procedure, nonadherent cells to a nylon-wool column (19) were fractionated in discontinuous Percoll gradients (20), and the cells with densities of $1.060 \leqq \rho < 1.070$ (large) or $\rho > 1.070$ (small) were collected and treated with a mixture of cytotoxic anti-I-A antibodies and either anti-L3T4 or anti-Lyt-2 antibodies, respectively, in the presence of rabbit complement. Alternatively, total spleen cells were directly treated with the appropriate mixtures of cytotoxic antibodies and complement before separation in a Percoll gradient. All of these cell populations were >98% Thy-1$^+$. Large and small T cells also were obtained by sedimentation at unit gravity (21) in continuous albumin gradients of anti-I-A and complement-treated spleen cells.

Cell Cultures and Assays. All cultures were set in 0.2-ml flat-bottom microtiter plates as described (16). Growth of purified small and large T lymphocytes was assayed in cultures of 10^5 cells in medium without or with Con A at 2.5 μg/ml or in conditioned medium containing IL-2. Proliferation was assessed by incorporation of tritiated thymidine (Amersham; specific activity, 5 Ci/mmol; 1 Ci = 37 GBq) after a 4-hr pulse with 1 μCi per culture.

Effector cell helper activity was assayed as described (6) by coculturing $4–5 \times 10^4$ anti-Thy-1- and complement-treated "indicator" cells with a titration of the various T-cell populations that were irradiated with 1200 rads, in the presence of Con A at 2.5 μg/ml. Effector cell suppressor activity was assayed by coculturing the same T cell-depleted spleen cell populations, this time stimulated by LPS at 25 μg/ml with appropriate titrations of the purified, irradiated T-cell preparations. In both cases, B-lymphocyte proliferative responses were measured by incorporation of tritiated thymidine as above, while the numbers of immunoglobulin-secreting cells were determined in the protein A plaque assay (22). Results shown were always the mean of triplicate cultures, for which

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Abbreviations: FACS, fluorescence-activated cell sorter; Con A, concanavalin A; LPS, lipopolysaccharide; IL-2, interleukin 2; PFC, plaque-forming cell; T_H cell, helper T cell; T_S cell, suppressor T cell; CTL, cytotoxic T lymphocyte(s).

Table 1. Activated (large and cycling) T and B cells in spleen of 12-wk-old unimmunized BALB/c mice

Marker	% of total spleen cells		% of large cells in each population	Cells in S + G_2 + M*		[^{3}H]Thymidine uptake in T cells, cpm per 10^5 cells	
	Large*	Small*		% total cells	% large cells	Large†	Small†
—	11.6–12.5	87.5–88.4	12.0	4.8–5.0	38–43	11,460	420
sIgM	4.8– 5.2	49.1–50.4	9.1				
L3T4	1.3– 1.4	18.3–19.5	6.7				
Lyt-2	0.9	7.3– 7.5	10.7				

sIgM, surface IgM.
*The results shown represent the range of values obtained in the analysis of two individual mice. Similar results were obtained in many other experiments.
†Separated by unit gravity sedimentation.

the SEM in every case was <10% of the mean. Lectin-dependent effector cell cytolytic activity was detected as described (7) in a 4-hr assay using ^{51}Cr-labeled target cells. The preparation of Con A-induced Lyt-2$^+$ cytotoxic effector cells and the preincubation of target cells with Con A have been described (23).

RESULTS

Quantitation of Activated (Large) and Cycling Cells in the Spleen of Normal Unimmunized Mice. As a first criterion for an operational definition of activated lymphocytes, we studied cell-size distribution by forward light scatter in the FACS. In such profiles, the majority of normal spleen cells appear to be in a single homogenous peak, with a median size slightly larger than erythrocytes. However, a defined fraction of the cells are readily separable on these bases and constitute medium- and large-size lymphocytes. Double-parameter analysis for size and fluorescence intensity in cell populations stained for surface expression of immunoglobulin, Lyt-2, or L3T4 markers allowed for quantitating the exact proportions of medium-to-large-size cells within each lymphocyte subset. The spleen of normal specific pathogen-free mice contains a population of large lymphocytes (Table 1) that constitutes ≈10% of the total. Furthermore, approximately the same frequencies of activated cells are represented in each of the lymphocyte populations defined by surface markers. These include B cells and the precursors for T_H and T_S/CTL lymphocytes, respectively. Many of these large cells are apparently in mitotic cycle, as evidenced by the FACS analysis of DNA content or by the "spontaneous" uptake of DNA precursors into the population of large cells purified by velocity sedimentation at unit gravity (Table 1). The numbers of cells in S + G_2 + M phases and the estimated length of G_1 in lymphocytes (24) allows the conclusion that roughly two-thirds of such large cells are engaged in the mitotic cycle. On the other hand, the spontaneous thymidine uptakes shown in the Table, while not suitable for quantitative determinations on the numbers of cells, do demonstrate that all cells in S phase are indeed large T lymphocytes.

The results demonstrate the "natural" occurrence of activated T cells in unimmunized mice, adding to previous and extensive descriptions of natural antibody levels and numbers of "background" Ig-secreting plasma cells in normal individuals (25). It would appear that equally large fractions of the B cell, T_H cell, and T_S/CTL cell pools are naturally activated (Table 1).

Naturally Activated (Large) T Cells Require No Induction To Grow in IL-2. A second criterion that can be used in the definition of activated T cells is the expression of IL-2 receptors, absent in the resting cells (26), which enable the activated cell to proliferate in the presence of the appropriate growth factor. This question was addressed by separating large and small cells from purified splenic T lymphocytes of normal mice and assaying their reactivity to IL-2. Large T cells directly initiated exponential growth in the presence of IL-2, while the small-cell fraction completely failed to proliferate with IL-2 alone and required activation with a polyclonal lectin (Fig. 1).

Large but Not Small T Lymphocytes from Normal Mice Are Effector T_H and T_S Cells. A third and definitive criterion for the definition of activated lymphocytes in the large population would be the expression of effector functions, that is, the equivalent in the T-cell compartment of plasma cell differentiation. As we do not know the clonal specificity of the activated cells, we could only address this question by taking advantage of two recently developed systems that allow detection of effector T_H (6) or T_S (unpublished data) cells, regardless of their specificities. For our purposes, such test should exclusively reveal the activity of effector T cells and not those of immunocompetent but resting T lymphocytes. Therefore, we have purified from the spleen of normal unimmunized mice populations of either L3T4$^+$ or Lyt-2$^+$ cells, separated each of these into small and large fractions, and titrated all four cell populations into cultures containing a fixed number of purified syngeneic B lymphocytes in the presence of either Con A or LPS. In the first case, helper

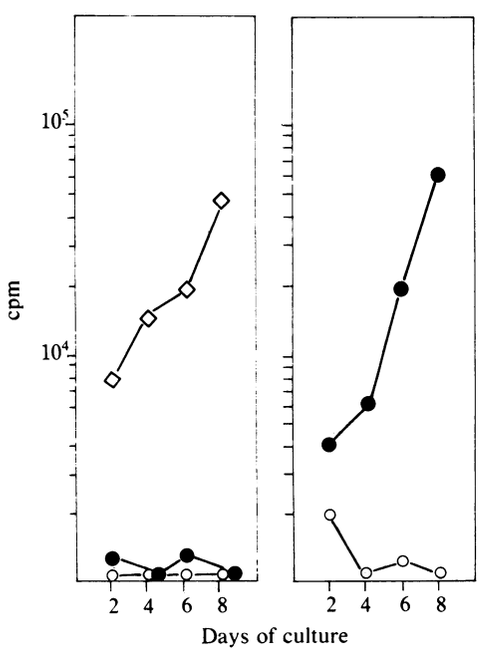

FIG. 1. Large (*Right*) but not small (*Left*) splenic T cells recovered from normal mice grow in IL-2-containing conditioned media. Cells were cultured in medium either without (○) or with Con A at 2.5 μg/ml (◇) or in IL-2-containing supernatants (●). The proliferative responses were assayed on the indicated days.

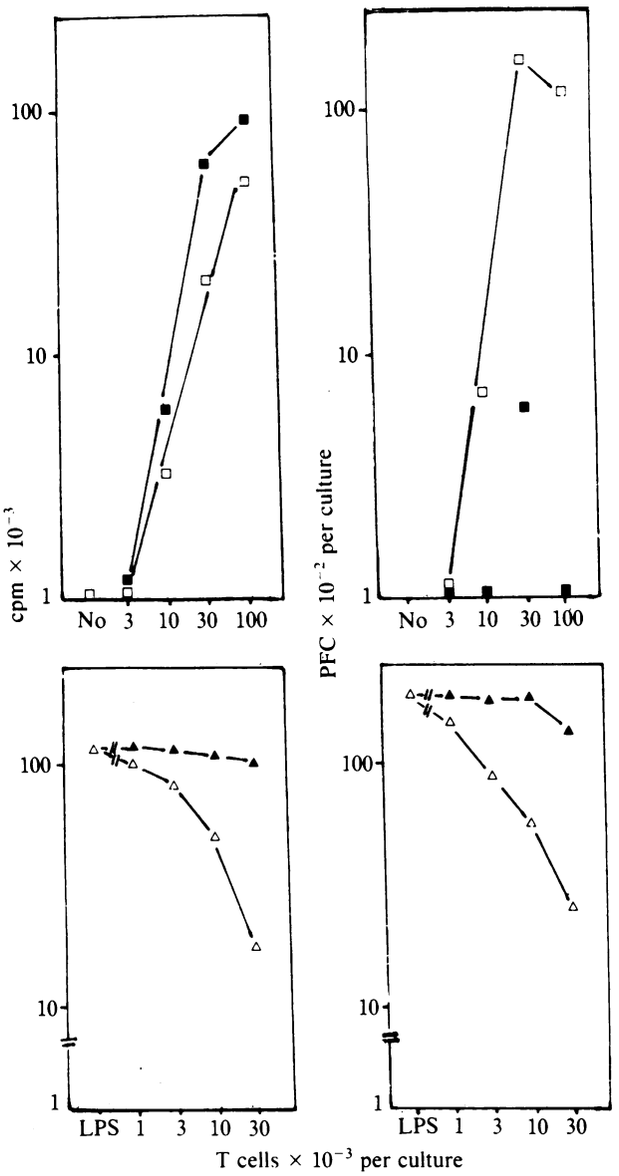

FIG. 2. Large but not small T lymphocytes from normal mice are competent T_H and T_S cells. Large (□) and small (■) L3T4$^+$ T cells were tested for helper activity (*Upper*), while large (△) and small (▲) Lyt-2$^+$ cells were assayed for suppressor activity (*Lower*). Proliferative responses were measured on day 3, and PFC were measured on day 5 of culture. No, no T cells added.

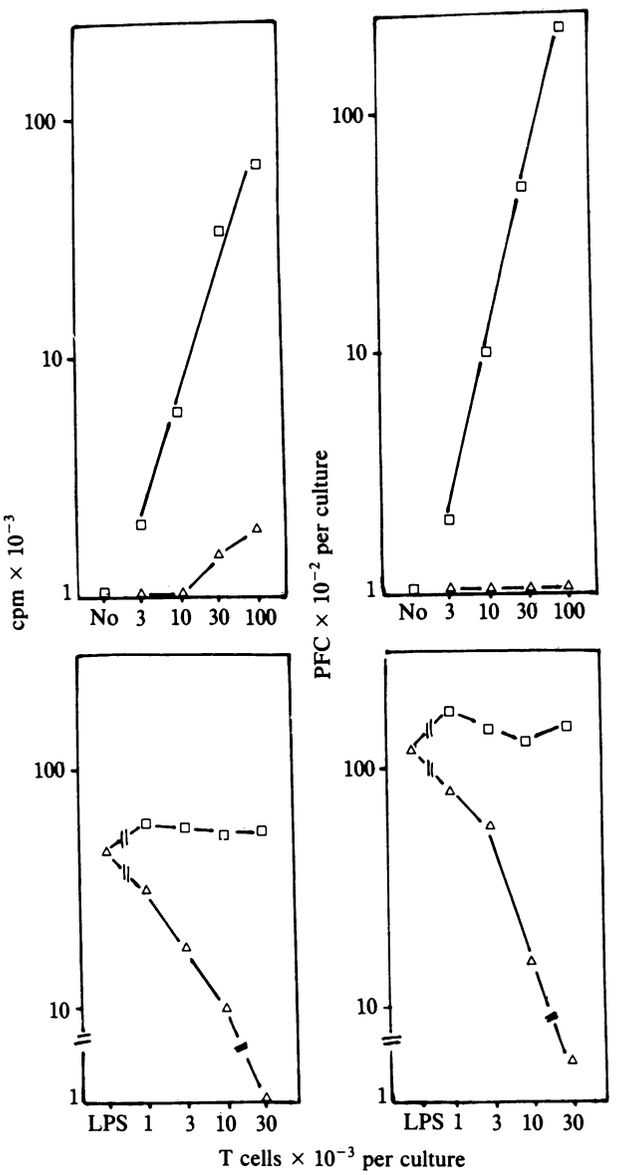

FIG. 3. Large L3T4$^+$ or Lyt-2$^+$ T lymphocytes selectively help or suppress. Irradiated large L3T4$^+$ (□) or Lyt-2$^+$ (△) T lymphocytes were tested in parallel for helper (*Upper*) and suppressor (*Lower*) activities. Proliferative responses were measured on day 3, and PFC were measured on day 4 of culture.

activity is selectively revealed by measuring proliferation or differentiation of the "target" B cells to plaque-forming cells (PFC) (6), while in the second case, the control responses to the B-cell mitogen LPS provide appropriate conditions for the detection of suppressive activities.

When Con A was added to the cultures allowing "nonspecific" collaboration, large L3T4$^+$ Lyt-2$^-$ T cells were fully competent in inducing growth and maturation to high-rate secretion of immunoglobulin in purified B cells, in contrast to small lymphocytes of the same phenotype, which are devoid of effector cell helper functions (Fig. 2 *Upper*). As the small T cells are perfectly competent and if not irradiated vigorously proliferate in these cultures (Fig. 2 *Upper Left*), these results demonstrate the selective expression of effector functions by the large T-cell populations.

The same general conclusion applies to effector T_S-cells. Large L3T4$^-$ Lyt-2$^+$ T cells were extremely efficient in suppressing the growth and maturation to PFC of B-cell populations stimulated with LPS, while small L3T4$^-$ Lyt-2$^+$ T cells are very much less efficient, if active at all, also in this respect (Fig. 2 *Lower*).

Surface Phenotype Segregation of Natural Effector T_H and T_S Cells. In order to cross-check the effector cell assays used above, we titrated populations of large L3T4$^+$ Lyt-2$^-$ or L3T4$^-$ Lyt-2$^+$ into both helper and suppressor assays. Furthermore, these T-cell populations were irradiated so that all proliferative activity in the test cultures could be ascribed to the indicator B lymphocytes. All effector cell helper activity was confined to the L3T4$^+$ Lyt-2$^-$ population, and the reverse applied to the cells mediating suppressor effects, which were present exclusively in the L3T4$^-$ Lyt-2$^+$ compartment (Fig. 3).

Naturally Activated T Cells Do Not Include Cytolytic Effector Cells. Another effector function of activated T cells is the lysis of target cells. For a number of years, a lectin-dependent polyclonal assay has been available that detects all CTL regardless of clonal specificities (7). To our surprise, however, activated Lyt-2$^+$ lymphocytes isolated from normal mice were completely devoid of cytotoxic activity in assays

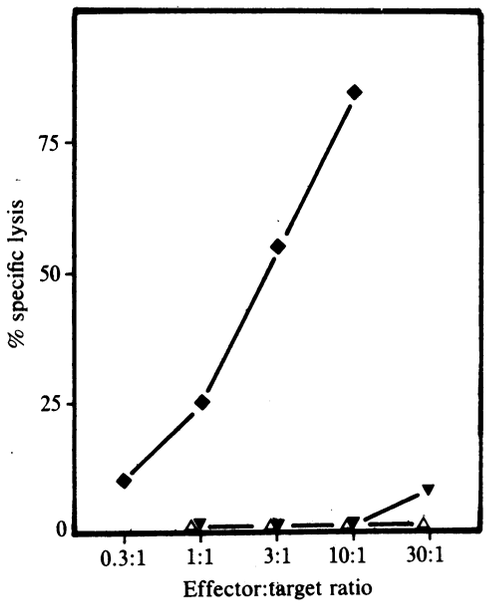

FIG. 4. Large T cells are not cytolytically active. Large Lyt-2$^+$ cells (▼) and total large T cells (△) were assayed for effector cell cytolytic function in a lectin-dependent killer assay using P815 target cells precoated with Con A. As a positive control, T-cell blasts derived by activation of small Lyt-2$^+$ cells with Con A (◆) were tested in parallel.

that readily reveal this effector function in irrelevant CTL populations (Fig. 4). This finding is all the more astonishing because the same cell populations are extremely efficient in suppressing B-lymphocyte responses (Figs. 2 and 3).

DISCUSSION

These results establish the presence of competent effector cells within the pool of large splenic T lymphocytes in normal nonimmunized mice, but they do not allow an appropriate quantitation of effector cell numbers. In similarity with the B-cell compartment, it might be expected that only a minority (5–10%) of all large activated cells are fully differentiated into effector functions. It is worth noting, however, the extreme efficiency of these cells in both suppressor and helper assays, contrasting with the complete absence of cytolytic activity. Thus, 1–3 × 10^3 L3T4$^-$ Lyt-2$^+$ large cells suffice to suppress 50% of the LPS-induced responses in 4 × 10^4 B lymphocytes, and the helper activity of the L3T4$^+$ Lyt-2$^-$ large cells compares well with that of T_H-cell lines that we have characterized (27, 28). It is all the more surprising, therefore, to find that no cytolytic activity is mediated by these cell populations, as if help and suppression of B lymphocytes were integral components of the normal immune physiology, whereas effector CTL would only develop in case of infectious pathology or under extreme conditions of intentional immunization with allogeneic cells or tissues. The physiological *in vivo* role of naturally activated T_H and T_S cells remains to be elucidated, but it is inescapable that they exert these activities in the normal immune system, much in the way as natural antibodies are one of its intrinsic components (29). Perhaps one of their functions would precisely be the regulation of natural antibody production and, consequently, their ultimate significance would be related to that of natural antibodies themselves.

This brings us to consider the mechanisms leading to the natural activation of this considerably large pool of lymphocytes in normal animals. These are exposed, of course, to microbial products and to a variety of other foreign antigenic materials at the mucosal surfaces. Moreover, normal immune systems are in permanent contact with most self antigenic patterns, those of the immune system itself constituting the largest diversity. All of these external or internal antigens may contribute to the natural activity reported here, but ongoing experiments with germ-free and antigen-free mice-axenic individuals fed low molecular weight diets indicate the internal or autonomous nature of this activity. Therefore, these findings are in contrast with current convictions that all mature lymphocytes in the immune system, if secluded from environmental stimuli, are small resting cells, and that all immune activity represents antigen-dependent immune responses. Rather, such naturally occurring internal activity would reflect some degree of functional autonomy in the immune system and constitute one of the essential characteristics of a system in a dynamic steady state, participating in the selection of repertoires, regulating the interactions among its own components, or even between itself and other components of the self. The study of the clonal specificity of the internally activated cells will help to evaluate these possibilities. In fact, repertoire analysis recently carried out for natural antibody-secreting cells has already indicated that they constitute a selected set of specificities (30). The internally activated lymphocytes may well constitute an interacting pool of cells, separable from the majority of lymphocytes, which remain resting and awaiting stimulation by external antigen.

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