Brief Definitive Report

L3T4 ANTIGEN EXPRESSION BY HEMOPOIETIC PRECURSOR CELLS

By GAY G. FREDRICKSON AND ROSS S. BASCH

From the Department of Pathology and Kaplan Cancer Center, New York University School of Medicine, New York, New York 10016

The CD4 antigen (murine L3T4) was originally described as a differentiation antigen expressed by the helper/inducer subset of peripheral T cells (1–3). A physical interaction between the molecule expressed by $CD4^+$ T cells and Ia antigen has been demonstrated that is strong enough for cell-cell binding to occur in the absence of other adhesion factors (4). The human equivalent is also believed to be part of the receptor for the HIV-I virus (5).

L3T4 expression is not restricted to thymocytes and mature T cells. The human Leu-3/T4 antigen has been found on monocytes (5), and 20–60% of mouse bone marrow cells also express the L3T4 antigen (2). Using positive selection, we now demonstrate that the $L3T4^+$ population of the bone marrow includes virtually all of the multipotential hematopoietic stem cells (CFU-s) and most of the myeloid precursors that proliferate in the presence of appropriate growth factors. These results suggest that L3T4 plays a role in cell interactions beyond that already demonstrated in the immune system.

Materials and Methods

Mice. Female mice, 5–6 wk old, were obtained from The Jackson Laboratory, Bar Harbor, ME.

mAbs. Phycoerythrin (PE)-conjugated anti-L3T4 (clone GK 1.5, rat IgG2b) was purchased from Becton Dickinson & Co. (Mountain View, CA). Anti-L3T4 (an ascites fluid from mice bearing the hybridoma YTS 191.5) was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY) as was FITC-coupled anti-Lyt-2 (clone YTS 169.4, rat IgG2b). The anti-L3T4 was biotinylated as previously described (6).

Staining and Analysis by Flow Microfluorometry. Immunofluorescence was performed as described previously (7). Fluorescence was quantified using a cytofluorograph (model 50H; Ortho Diagnostic Systems Inc., Westwood, MA) equipped with a 5-W argon laser operating at 200 mW at 488 nm. Sorgin and analysis were performed under the same conditions.

CFU-s Assay. CFU-s were determined using a modification of the method of Till and McCulloch (7, 8). BM cells (10^5) from B6D2.F1 mice were injected intravenously into irradiated (850 rad) syngeneic mice. A ^{137}Cs source (model M; Gammator Radiation Machinery Corp., Parsippany, NJ) delivering 430 rad/min was used for irradiation. On day 8 and 12 after irradiation, spleens were removed, fixed in Bouin's solution, and the macroscopically visible colonies counted.

CFU-c Assay. Marrow cells forming colonies in semi-fold agar were assayed as previously described (6). L cell-conditioned medium was added to all cultures as a source of CSF (9).

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Address correspondence to Ross S. Basch, Department of Pathology, N. Y. U. Medical Center, 550 First Avenue, New York, NY 10016.

Cell Cycle Analysis. Bone marrow cells were stained with biotinylated anti-L3T4 and fluoresceinated avidin and then their DNA was stained with propidium iodide as described by Deitch et al. (10). Correlated two-color flourescence data was collected in list mode. G_0/G_1, S, and G_2/M phases were determined graphically and then the data reanalyzed to determine the proportion of antigen-positive cells at each stage of the cell cycle.

Results

L3T4 Antigen Expression by Bone Marrow Cells. Fig. 1 depicts a contour plot of mouse bone marrow stained with PE-conjugated anti-L3T4 antibody. Significant staining of bone marrow cells was found. The extent of L3T4 expression varied over a range of 19–65% of the nucleated cells (mean of 34.6 ± 15.3 in BDF_1 animals)[1]. The staining pattern did not appear to be age, sex, or strain related (6 wk to 22 mo; AKR/J, C57BL/6, $B6D2F_1$, and BALB/c). The mean fluorescence intensity of the antigen-positive cells in the marrow was 1/6th that of cortical thymocytes stained under similar conditions.

To assess the developmental potential of these cells, $L3T4^+$ and $L3T4^-$ cells were selected by sorting (Fig. 1, insert), and were either cultured in semi-solid agar or injected into irradiated mice. Cells with high right angle light scatter (developing granulocytes [11]) were excluded from sorts in which CFU-s were being measured.

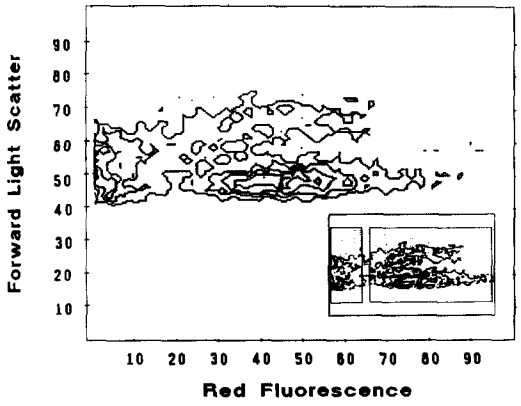

FIGURE 1. L3T4 expression of AKR/C bone marrow. Two-parameter histogram (contour plot) of forward light scatter vs. fluorescence intensity. The analysis and subsequent sort were performed using a cell sorter (Ortho Diagnostic Systems Inc.). The inset shows the gating used to establish the windows used to sort $L3T4^+$ and $L3T4^-$ BM cells. The region containing antigen-negative cells was established using unstained bone marrow cells since no irrelevant PE-coupled IgG2b rat mAb was available. Experiments in which antigen-positive cells isolated after staining with an FITC-coupled IgG2b rat mAb (anti-CD8, clone YTS 169.4) did not produce a similar staining pattern and did not yield enrichment of CFU-s.

TABLE I

Myeloid Precursor Activity (CFU-c) of $L3T4^+$ and $L3T4^-$ Bone Marrow Cells

Cells cultured	Colonies*
Sorted $L3T4^-$	7.3 ± 3.1
Sorted $L3T4^+$	57.8 ± 7.9
Sorted unselected	19.3 ± 2.0

* Mean number of colonies (± SD) observed on day 6 per 6×10^4 BDF_1 BM cells cultured in semi-solid agar. Reanalysis of the $L3T4^+$ cells indicated a purity of 96.7%. The $L3T4^-$ population reanalyzed as >98% negative. Only colonies containing at least 30 cells were scored. Four replicate cultures were performed for each experiment. Unstained marrow cells produce 28.3 ± 6.3 colonies per 6×10^4 cells when cultured under these conditions. Passage through the sorter results in a 15–20% loss of CFU-c.

[1] Using indirect immunofluorescence (biotinylated YTS 191.5 and PE-streptavidin), the mean number of $L3T4^+$ cells in BDF_1 mice is 52.8 ± 9.3%).

TABLE II

Spleen Colony Forming Activity (CFU-S) of L3T4$^+$ and L3T4$^-$ Bone Marrow Cells

	Colonies*	
Cells injected	Day 8	Day 12
Sorted L3T4$^-$	1.3 ± 0.9	2.5 ± 1.5
Sorted L3T4$^+$	11.3 ± 4.0	17.3 ± 2.1
Sorted unselected	9.7 ± 3.9	9.2 ± 4.5
No cells injected	0	1.5 ± 1.5

* Mean number of 12-day spleen colonies per 10^5 BDF1 BM cells injected into irradiated (850 rad) syngeneic mice (± SD). Reanalysis of the positive cells indicated a purity of >97%, while reanalysis of the negative indicated that they were >98% pure. Unstained, unsorted cells produce 10.1 ± 2.1 colonies at 8 d and 11.4 ± 3.1 colonies at 12 d.

All nucleated marrow cells were included in the sorts for CFU-c activity. Table I shows the distribution of myeloid precursors in the sorted populations. In this experiment 37.7% of the nucleated cells were L3T4$^+$. The majority of cells that were capable of growing in semi-solid agar in the presence of CSF-1 were found in the L3T4$^+$ fraction. The precursor activity of the brightest 35% of the L3T4$^+$ population was not significantly different than the total L3T4$^+$ population, indicating that this activity is attributable to cells with a heterogenous expression of the antigen (data not shown).

The data in Table II show that virtually all of the cells able to form spleen colonies, at either 8 or 12 d after transfer, are also L3T4$^+$. The results shown are from a single experiment in which 31.8% of the marrow cells were L3T4$^+$, but are representative of many such separations. Mixing the cells expressing the most L3T4 antigen (L3T4 bright) with negative cells does not produce the synergistic effect that would be expected if the L3T4$^+$ cells exerted a "helper" function that enabled the L3T4$^-$ cells to produce colonies. The gates used to separate L3T4$^+$ and L3T4$^-$ cells exclude the dimly L3T4$^+$ population that comprises 10–25% of the cells of the

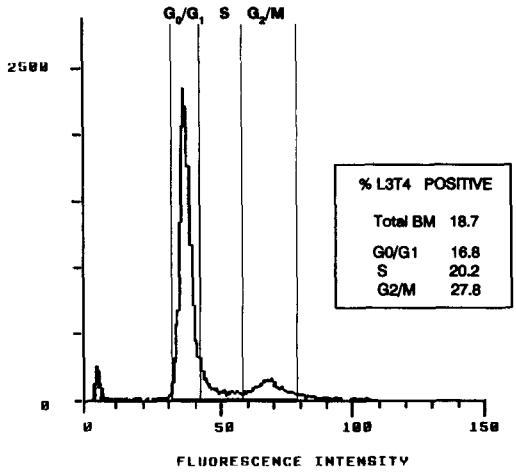

FIGURE 2. Relationship of L3T4 expression to position in the cell cycle. Bone marrow cells were stained with biotinylated anti-L3T4 and fluoresceinated strepavidin and then stained with propidium iodide. The samples were then analyzed on a FACScan flow cytometer. Separate gate sets were established for the G_0/G_1, S, and G_2/M populations, as well as for the entire sample.

marrow. These cells have CFU-s activity equal to that of the L3T4 bright population (data not shown).

L3T4 Expression Is not Restricted to a Specific Stage of the Cell Cycle. Fig. 2 illustrates the DNA histogram obtained when murine bone marrow cells (BDF_1) are stained with propidium iodide after immunofluorescent staining for L3T4 (with a biotinylated antibody and fluoresceinated strepavidin). The total sample contained 18.7% $L3T4^+$ cells. The proportion of $L3T4^+$ cells at each stage of the cell cycle is also shown in the figure. The mitotically active cells (S, and G_2/M) are somewhat more antigen positive than those in the G_0/G_1 phase, but expression is clearly not restricted to any particular phase of the cell cycle.

Discussion

We have shown that the L3T4 marker, usually associated with Th cells, is present on a substantial proportion of murine hematopoietic precursors. Virtually all 8- and 12-d CFU-s are $L3T4^+$, as are the majority of the CSF-1-dependent CFU-c.

The finding that L3T4 is expressed on cells outside of the T lineage is not surprising. L3T4 and its human analogue Leu-3/T4 are known to be present on developing myeloid cells. The original report describing GK1.5, an mAb-specific for L3T4, indicated that many mouse bone marrow cells were stained by the reagent (2). The cells of the myeloid series, like those of the T lineage, lose the antigen as they mature, although low levels of the human analogue are retained by circulating monocytes (5, 12).

It is surprising that early progenitors and multipotential stem cells are $CD4^+$. Spangrude et al. (13) have recently reported the isolation of murine multipotential cells using a protocol that was said to remove $CD4^+$ cells. The extent to which $CD4^+$ cells were depleted was not measured directly and the technique used (magnetic beads coated with anti-Ig were used to remove cells coated with anti-CD4 antibodies) is both affinity and concentration dependent. It is effective only if the target cell expresses high levels of surface antigen (14). Our results indicate the hematopoietic precursors express much less antigen that do mature T cells. The evidence for the presence of L3T4 on CFU-s and CFU-c is unequivocal. Sorted positive cells produce colonies far better than do sorted negative cells. Fc binding is an unlikely explanation for these results since other antibodies do not produce similar results and FcR^+ B cells are not stained by the PE-coupled anti-L3T4. Antigen expression is not restricted to any phase of the cell cycle. The absence of activity in the $L3T4^-$ population is not due to a loss of a helper activity by which $L3T4^+$ cells facilitate the activity of the $L3T4^-$ population since: (*a*) the positive cells themselves possess high levels of CFU-s and CFU-c activity; and (*b*) mixing $L3T4^+$ and $L3T4^-$ cells does not produce the synergy that would be expected if one population were providing "help" for the other.

L3T4 is believed to be one of the accessory molecules that make up the TCR complex, interacting with Ia antigen on APC. Antibodies to CD4 inhibit lectin-induced lymphokine production and interfere with antigen-driven proliferation (15–18). These results have been interpreted as indicating that L3T4 serves either as a binding factor, facilitating cell-cell interaction, or as a receptor, transducing a signal that regulates cell activation. We believe that it could serve a similar role in interactions occurring during hematopoietic development. The interaction of an $L3T4^+$ lymphohemato-

poietic precursor in a hemopoietic inductive microenvironment (19) with an Ia^+ stromal cell would provide the means of either transferring a hemopoietic growth factor(s) or regulating the responsiveness of the target cells. L3T4 might, of course, react with other ligands as well as Ia.

Other potential roles for L3T4 on developing hematopoietic cells should be considered. The interaction of L3T4 molecules on the immature cells with Ia expressed by a stromal cell could be necessary to retain precursors in the marrow. The loss of L3T4 during development might be required for the cells to emerge into the circulation.

Summary

L3T4 (CD4) is expressed on immature hematopoietic cells. Sorting bone marrow cells on the basis of their expression of this antigen produces populations of cells that are markedly enriched for multipotential stem cells (CFU-s) and for myeloid precursors (CFU-c). We believe that L3T4 is transiently expressed by most, if not all, hematopoietic precursors early in their maturation. We suggest that the expression of CD4 molecules on the surface of immature precursors is required for their interaction with Ia bearing cells within the hemopoietic inductive microenvironment(s) of the marrow and thymus.

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