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PHENOTYPE OF THYMIC LYMPHOMAS IN THE MOUSE*

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Abstract—The MP2 cell line was established from a murine leukemia virus-induced thymic lymphoma. Half of the cells were consistently L3T4 positive and less than 5% of the cells were Lyt-2 positive. Single cell cloning on the basis of the presence or absence of Lyt-2 allowed the isolation of four clones with stable phenotypes: (1) Lyt-2$^-$, L3T4$^-$; (2) Lyt-2$^+$, L3T4$^+$; (3) Lyt-2$^-$, L3T4$^+$; (4) Lyt2$^+$, L3T4$^-$.

These data are discussed in relation to tumour cell heterogeneity and to normal T-cell differentiation pathways.

Key words: T-lymphocyte, lymphoma, differentiation, cytofluorimetry.

INTRODUCTION

WE HAVE recently reviewed the biology of thymic lymphoma in the mouse [1]. The following conclusions were proposed:

(a) tumour cells are *bona fide* T lymphocytes; there is no lineage infidelity;

(b) lymphomas corresponding to every stage of normal differentiation have been identified. Chemical carcinogens, X-rays and murine leukemia viruses (MuLV) induce the whole spectrum of T-cell tumours. There is practically no way to identify the agent used for induction;

(c) to a large extent, the phenotype of tumour cells is "frozen" at a given stage of differentiation.

Establishing the phenotype and ascertaining the differentiation level of murine lymphomas is not an academic exercise. This information is badly needed if murine lymphoma is to be taken as a model for the study of normal lymphocyte differentiation and leukemogenesis [1]. For these studies, both fresh tumours and tumour-derived cell lines have their respective advantages and potential pitfalls. Fresh tumour cell suspensions are contaminated by non-tumour cells. This is not the case with established lines. However, considerable selection occurs during the establishment of cell lines [2]. In addition, the loss of markers and the generation of variants in culture are well documented [1, 3]. Looking at either fresh tumours or cell lines, we were surprised by the fact that biphasic curves were occasionally generated when the expression of cell surface antigens was examined at the fluorescence-activated cell sorter (FACS).

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Abbreviations: *FACS*, fluorescence-activated cell sorter; *FITC*, fluorescein-isothiocyanate; *GaRIg*, goat anti-rabbit immunoglobulin antibody; *IL-2-R*, receptor for interleukin-2; *MuLV*, murine leukemia virus; *PNA*, peanut agglutinin; *RadLV*, radiation leukemia virus.

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MATERIALS AND METHODS

Cells

Radiation-induced lymphomas were obtained by four weekly irradiations (1.75 Gy) during the second month of life [3] in female C57Bl/Ka mice raised in our colony. Cell suspensions were prepared from thymic tumours of moribund animals.

The MP2 tumor was induced by intrathymic inoculation of radiation leukemia virus RadLV-MuLV as described [3] in a 30 day-old female C57Bl/Ka mouse. A cell line was established from the resulting tumor as explained in [3]. The present study was performed after about 50 *in-vitro* passages.

Monoclonal antibodies and other reagents

The fluorescein isothiocyanate (FITC) conjugates of monoclonal anti Thy-1.2 (clone 30-H-12 [14]) and anti Ly-1 (clone 53.7.3 [4]) were purchased from Becton Dickinson (Sunnyvale, CA). Other monoclonal antibodies were used in conjunction with FITC-conjugated F(ab')$_2$ fragments of goat anti-rat Ig

(FITC-GaRIg) obtained from Cappel, Worthington diluted 1:40. They include:

—anti-Lyt-2 (53–6.7) [4], produced by the cell line obtained from the Salk Institute (San Diego, CA).

—anti-L3T4 (H-129.19–6.8), produced by the rat hybridoma kindly provided by Dr M. Pierres [5].

—anti-interleukin 2-receptor (IL-2-R) (7D4) produced by the hybridoma line obtained through the American Tissue Culture Collection (Rockville, MD) [6].

—anti-IL-2-R (AMT-13), a gift from Dr T. Diamanstein [7]. The plain culture supernatant was used.

—anti-Pgp-1 (1M7.8.1), kindly provided by Dr J. Lesley [8].

Except for AMT-13, culture supernatants were made 50% in ammonium sulphate and the precipitate was dialyzed against phosphate-buffered saline. Peanut agglutinin (PNA) and D-galactose were purchased from Sigma (St Louis, MO) and used as described [9].

Flow cytometry and single-cell cloning

Flow cytometry was done as described [9]. Briefly, for each condition, 5000 cells were analysed with a velocity of 1000 cells/s using a FACS IV cytofluorimeter (Becton Dickinson, Mountain View, CA) equipped with a 70-μm nozzle and an argon ion laser emitting a 200-mW beam at 488 nm (type 164-05, Spectra Physics). On the basis of the gated forward-light scatter emission signal, the fluorescence emission of the cells of interest was collected using a 520-nm long-pass dielectric filter and a 530-nm long-pass glass filter in front of a photomultiplier (750 V, model 9524 A QL-30, Emi-Gencom Inc.) equipped with a three-decade logarithmic amplifier. Data were recorded on histograms with 256-channels resolution.

The cloning experiment was performed using the single-cell deposition system (Becton Dickinson) linked to the FACS. Cells were stained for Lyt-2 as mentioned above. Single Lyt-2$^+$ or Lyt-2$^-$ cells were electronically sorted by the FACS and deposited into wells (1 cell per well) of 96-well Nunclon microculture trays containing 200 μl of culture medium per well.

RESULTS

1. Typing

Table 1 shows the surface phenotype of cells obtained from radiation-induced thymic tumours L1 and L2. Figure 1 also clearly shows that L1 cells were heterogeneous with respect to quantitative expression of the L3T4 antigen and the receptor for IL-2. Actually, two distinct populations were easily identified in each case. The same holds true for the MP2 line: about half of the cells only expressed detectable levels of L3T4. Also, very few cells expressed Lyt-2.

2. Cloning

Single cell sorting on the basis of Lyt-2 expression and culture allowed the production of four clones, with phenotypes reported in Table 2 and illustrated in Fig. 2.

These phenotypes remained stable during several passages (R.G., in preparation).

Twenty other clones were generated in the same experiment. Their characterization is in progress.

TABLE 1. SURFACE PHENOTYPE OF LYMPHOMAS AND CELL LINES

Cells	Thy-1	Ly-1	Lyt-2	L3T4	IL-2-R	Pgp-1	PNA
L1	96*	46	67	60‡	20§	19	ND†
L2	93	ND	49	24	ND	ND	45
MP2	95	0	4	55‡	77‡¶	0	8–28

* Percent positive cells.
† Not done.
‡ Bi-modal curve.
§ Staining with AMT-13 monoclonal antibodies.
¶ Staining with 7D4 monoclonal antibodies.

TABLE 2. SURFACE PHENOTYPES OF CLONES DERIVED FROM THE MP2 LINE

	Lyt-2	L3T4	IL-2-R†	PNA
Clone 1	<1*	<1	100	94
Clone 2	95	97	80‡	91
Clone 3	<1	100	90‡	60
Clone 4	100	5§	100	88

* Percent positive cells.
† Staining with 7D4 monoclonal antibodies.
‡ Bi-modal curve.
§ Low intensity.

DISCUSSION

Although the MP2 line contains only a few Lyt-2$^+$ cells, four cell types, at least (corresponding to clones 1 to 4), are probably present in the uncloned line. It is, for instance, possible that an immature cell (Lyt-2$^-$, L3T4$^-$) has been transformed but kept its potential to differentiate, mimicking the normal T-cell differentiation pathway [10–14]:

$$\text{Lyt-2}^-, \text{L3T4}^- \rightarrow \begin{cases} \nearrow \text{Lyt-2}^+, \text{L3T4}^+ \\ \rightarrow \text{Lyt-2}^+, \text{L3T4}^- \\ \searrow \text{Lyt-2}^-, \text{L3T4}^+ . \end{cases}$$

For unknown reasons, the Lyt-2$^-$ cells are poorly represented in the uncloned line. Alternative pathways are, however, suggested by our recent observations (R.G., in preparation). After the culture conditions were changed, some cells from both Lyt-2$^-$ clones expressed Lyt-2:

$$\text{Lyt-2}^-, \text{L3T4}^- \rightarrow \text{Lyt-2}^-, \text{L3T4}^-$$
$$\text{Lyt-2}^-, \text{L3T4}^+ \rightarrow \text{Lyt-2}^+, \text{L3T4}^+ .$$

The following scheme would also be compatible with our data. However, it does not fit in with proposed differentiation pathways [10–14]

$$\text{Lyt-2}^-, \text{L3T4}^- \begin{cases} \nearrow \text{Lyt-2}^+, \text{L3T4}^- \\ \searrow \text{Lyt-2}^-, \text{L3T4}^- \\ \qquad \searrow \\ \qquad \text{Lyt-2}^+, \text{L3T4}^- . \end{cases}$$

In contrast, classical observations on the loss of cell-surface antigens in culture [3] would favor the idea that the original tumor expressed both Lyt-2 and L3T4. Subpopulations lacking one or both markers could have

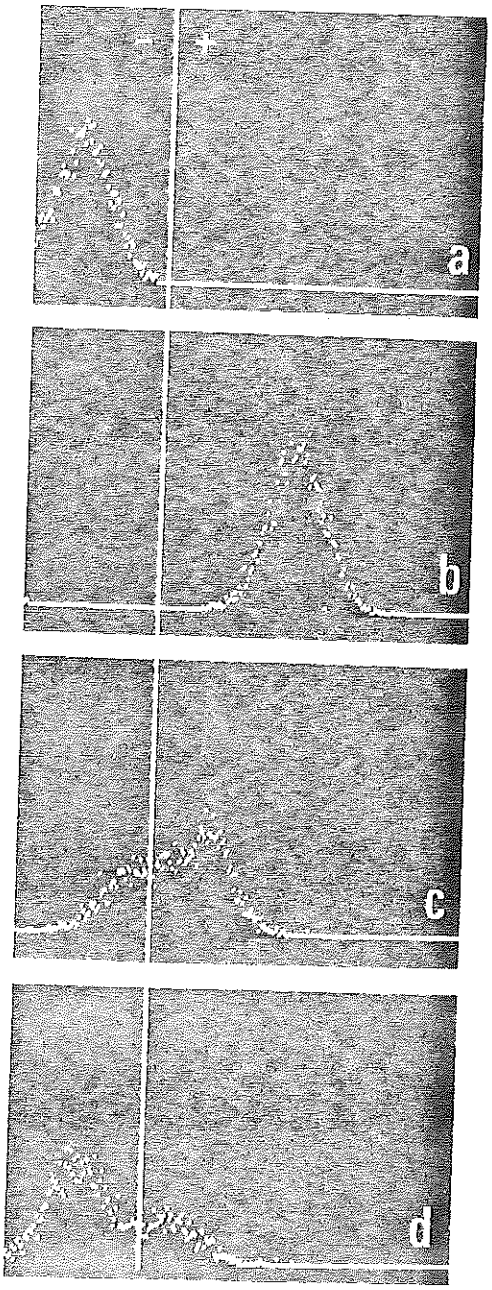

FIG. 1. FACS analysis of fresh tumour cells from lymphoma L1, stained with anti-Thy 1.2 (b), anti-L3T4 (c), anti-IL-2-R (d), or FITC-GaRIg only (a). Vertical axis: relative cell number. Horizontal axis: relative fluorescence intensity (log scale). Percentage of positive cells is given in Table 1. Curves are clearly bi-modal with antibodies to L3T4 and to the IL-2 receptor.

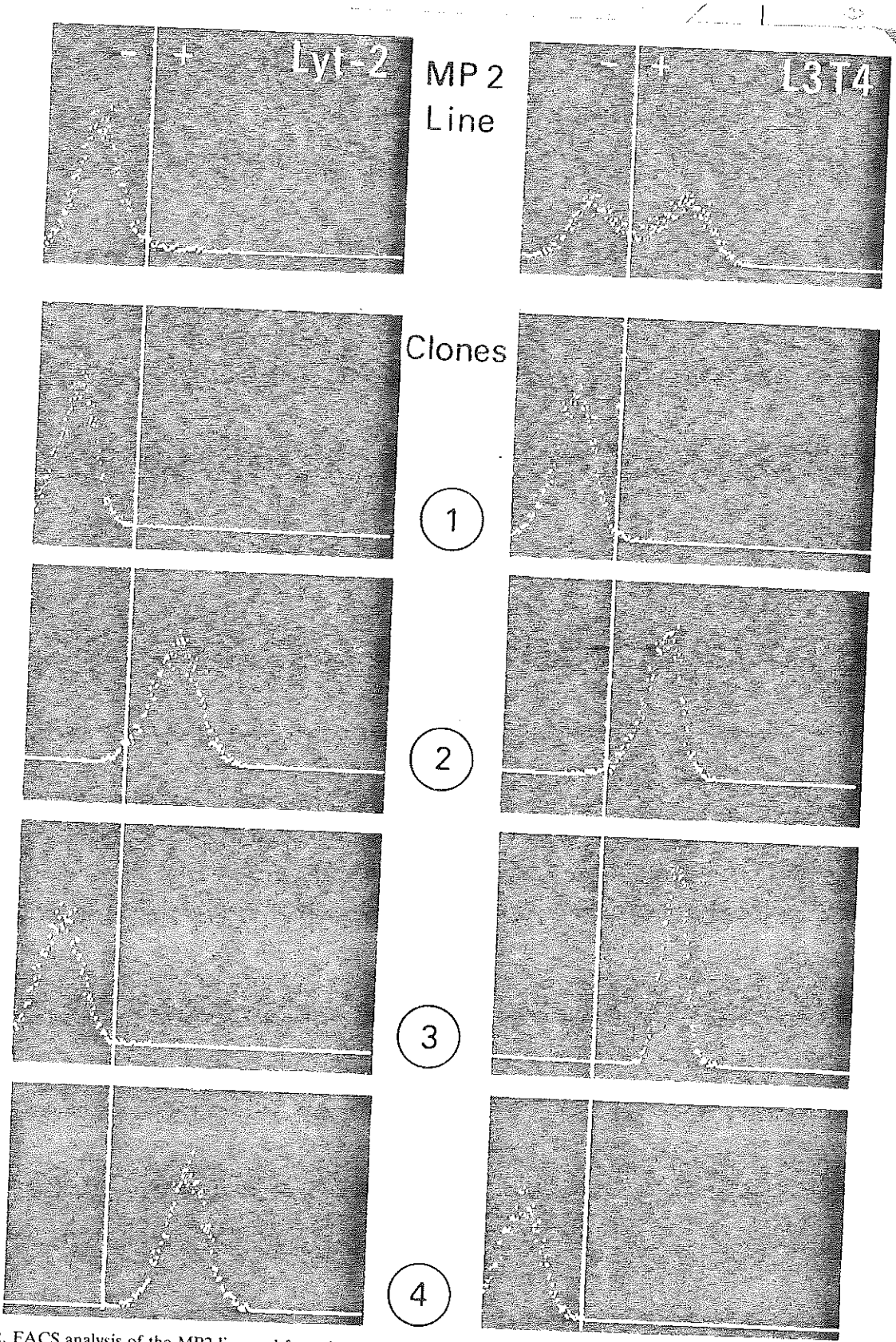

FIG. 2. FACS analysis of the MP2 line and four clones derived from it on the basis of expression of Lyt-2 (clones 2 and 4) or lack of it (clones 1 and 3). Cells were stained with anti Lyt-2 (left) or with anti L3T4 (right). See Table 1 for percentage of positive cells. The MP2 line expresses a low level of Lyt-2 and half of the cells are L3T4 positive. The $L3T4^-$ cells clearly belong to a distinct peak. Clone 1: $Lyt\text{-}2^-$, $L3T4^-$. Clone 2: $Lyt\text{-}2^+$, $L3T4^+$. Clone 3: $Lyt\text{-}2^-$, $L3T4^+$. Clone 4: $Lyt\text{-}2^+$, $L3T4^-$. Vertical and horizontal axes as in Fig. 1.

arisen during *in-vitro* passages. Re-expression of lost markers is also well documented [1, 3].

Obviously, it is now critical to test whether the MP-2 line is clonal in origin. Virus integration site is a good marker for clonality [15, 16].

Our observations showing cellular heterogeneity in murine lymphomas are in keeping with recent publications but in sharp contrast with classical data. Indeed, most studies, including some recent work done with monoclonal antibodies and FACS, yielded unimodal curves only [17]. However, O'Donnell *et al.* often found two populations in virus-accelerated lymphomas using different markers, most commonly Lyt-2 [18]. The pattern was conserved upon transplantation, suggesting that both populations were malignant and stable. Yetter and Morse [19] also described several lymphomas induced in the same way and containing 41–66% Lyt-2^+ cells. One tumour was transplanted into four mice. One of these developed a Lyt-2^- tumour whereas three mice had tumours with 30–55% Lyt-2^+ cells [19]. The fact that cells with two different phenotypes were found in a single tumour does not prove that the tumour was not monoclonal in origin. There is indeed considerable evidence now that heterogeneity can be generated after transformation [1, 20].

Following classical pathological observations, it was agreed that all thymic lymphomas in the mouse were very similar [21]. Since 1978 however, many reports had shown that nearly any (normal) phenotype could be generated by any protocol of lymphoma induction, provided that enough mice were treated [22–24]. In contrast, several laboratories have reported quite recently evidence suggesting that a given agent favours the induction of one particular phenotype. For instance, Scott *et al.* have shown that radiation-induced lymphomas more often express the Ly-1^-, Lyt-2^+ pattern whereas RadLV-MuLV-induced tumours preferentially express the Ly-1^+, Lyt-2^- phenotype [17]. Newcomb *et al.* claim that virus-induced tumours were more differentiated than radiation-induced lymphomas. Indeed the latter consistently expressed TL, Ly-1 and Lyt-2 and the enzyme terminal deoxynucleotidyl transferase [25]. Goodenow and Lilly, on the other hand, oppose spontaneous lymphomas in the AKR strain to methylcholanthrene-induced tumours in RF mice [26]. On the basis mainly of Thy-1 and H-2 expression, they state that the spontaneous tumours correspond to immature, cortical thymocytes whereas the carcinogen-induced lymphomas could be neoplastic equivalents of cells involved in feed-back suppression of antibody (low Ly-1, high Lyt-2, high H-2 and Qa1^+). Several workers have claimed that different MuLV's induce tumours with different phenotypes [27]. For instance, lymphoma cells have a cortical phenotype after induction with Abelson MuLV combined with Moloney MuLV as opposed to a medullary phenotype when RadLV-MuLV is used as helper for Abelson MuLV [28]. In virus-accelerated tumours of AKR mice, Yetter and Morse found no evidence suggesting that individual clonal mink cell focus viruses induce tumours of a unique antigenic phenotype [19], in contrast to Zielinski's earlier claim [27]. Finally, it may be worth mentioning that, so far, functional tumours were successfully induced with RadLV-MuLV's only [1]. We are, however, not aware of efforts to induce functional tumours with other agents. We have immunized several groups of mice at various times during radio-leukemogenesis but we could not find a single tumour with helper activity (R. H. and N. Schaaf-Lafontaine, unpublished results).

In view of the data reported recently by other investigators and by ourselves, we propose the following conclusions:

(a) Thymic lymphoma cells are *bona fide* T-cells. There is no evidence for lineage infidelity, nor is there any convincing sign that hemopoietic stem cells (or cells that are not committed to the T cell-lineage) would be consistently transformed in T-cell lymphomas (in contrast to recent theories on human B-cell malignancy [29]).

(b) It remains very hard to stage lymphoma T cells. Indeed, some normal populations, including pro-thymocytes and T-blasts are poorly characterized.

(c) Lymphoma T-cells lose or acquire differentiation markers after transformation. Tumour lines such as MP-2 are useful tools to analyse cellular and humoral factors controlling T-cell differentiation. The strategy for such studies could rely on elegant work in developmental neurobiology [30, 31].

Available evidence suggests that these conclusions also apply to human T-cell malignancies.

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