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Growth factors involved in lymphocyte differentiation

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[Plate 1]

We report here that an interleukin-3-dependent precursor B-cell line, LyD9, differentiated *in vitro* into mature B cells, producing immunoglobulin (Ig)M and IgG by co-culture with bone marrow stroma cells. Induced LyD9 cells underwent heterogenous immunoglobulin gene rearrangement and synthesized mRNAs encoding immunoglobulin mu (μ), gamma (γ) and kappa (κ) chains. LyD9 was also shown to differentiate into myeloid cells. We have established an interleukin-4-dependent derivative clone K-4 that is an intermediate between myeloid–lymphoid cells and the LyD9 clone. This differentiation required direct contact between LyD9 and stromal cells.

Introduction

T and B lymphocytes are believed to differentiate from a common precursor cell that is derived from a bone marrow stem cell. One of the most interesting features of lymphocyte differentiation is its association with DNA rearrangement in the immunoglobulin (Ig) loci of B lymphocytes or in the T-cell receptor loci of T lymphocytes. The V–D–J recombination that brings the variable (V), diversity (D) and joining (J) segments together to form a complete V gene is a decisive step of the B- or T-cell differentiation and thus serves as a genetic marker of commitment to either B or T lineage (Tonegawa 1983; Honjo & Habu 1985).

To investigate regulation of the decisive step of B-cell differentiation, it is important to establish an *in vitro* system that allows induction of the V–D–J and V–J recombination in a cell line containing the germ line context of the immunoglobulin gene. Establishment of interleukin (IL)-3-dependent progenitor cell lines, each of which can differentiate *in vivo* into either B or T lymphocytes (Palacios & Steinmetz 1985; Palacios & Pelkonen 1988) provided unique model systems for molcular biological studies on cellular commitment.

We report here the IL-3-dependent LyD9 progenitor clone has differentiated into mature B cells and myeloid cells *in vitro* when co-cultured with bone marrow stromal cells. We have established an IL-4-dependent clone (K-4) that was derived from induced LyD9 cells. The K-4 clone is a multi-potential intermediate in differentiation from the LyD9 clone to B cells and myeloid cells.

Materials and methods

Animals and cells

Interleukin (IL)-3-dependent cell lines (LyD9) were established from a 4-week-old CBA/J mouse (Palacios *et al.* 1987). ST-2 stromal cell line (Ogawa *et al.* 1988) established from Whitlock–Witte type bone marrow culture was provided by Dr S. Nishikawa (Kumamoto University).

Induction with bone marrow stromal cells

Bone marrow stromal cells were prepared as described by McKearn *et al.* (1985). LyD9 cells (1×10^5) were mixed and co-cultured with bone marrow stroma cells (5×10^5) (see Kincade *et al.* 1976). After 10 days of culture, cells were harvested for immunofluorescence staining with polyclonal rabbit anti-murine IgM or IgG antibody or with MB86 monoclonal anti-μ chain antibody (Nishikawa *et al.* 1986).

Establishment of K-4, K-GM and LS-1 cells

Induced LyD9 cells (5×10^5) that had been co-cultured with bone marrow stromal cells were collected and recultured in complete medium containing 1 mg ml^{-1} G418 (Sigma) supplemented with 10 U ml^{-1} rIL-4 or 20 U ml^{-1} rGM-CSF. Every third day rIL-4 or rGM-CSF was added to the culture medium and the outgrowth of cells became apparent in two weeks. The LS-1 clone was established as follows: induced LyD9 cells (5×10^5 ml^{-1}) were transferred and maintained on a monolayer of ST-2 cells in complete medium supplemented with 25 μg ml^{-1} lipopolysaccharide (LPS) and 1 mg ml^{-1} G418. After 4 weeks, the growing cells were subcloned by using a limiting dilution procedure in round-bottomed microtitre plates precoated with ST-2 cells. One of the outgrown cells (LS-1) was expanded and maintained on the monolayer of ST-2 in complete medium supplemented with 25 μg ml^{-1} LPS.

Morphological analysis

Cytocentrifuge preparations of cells were stained with May–Grünwald–Giemsa. Non-specific esterase granules were detected with α-naphthyl butyrate or chloroacetate staining methods. Peroxidase granules were detected as described (Goud *et al.* 1975).

Results

Differentiation of LyD9 cells by co-culture with bone marrow stroma cells

We co-cultured LyD9 cells with bone marrow stromal cells. LyD9 cells were loosely associated with the stromal cells and grew on their surfaces. After 10 days of culture, LyD9 cells formed a monolayer sheet of cells over the stromal cell layer, and the number of floating cells increased. Few floating cells appeared without the addition of LyD9 cells. The co-cultured LyD9 cells were analysed with flow cytometry after staining with antibodies against IgM, B220 and Thy 1.2. The expression of the B 220 antigen increased during co-culture (figure 1*a*), but no Thy 1.2-positive cells appeared (figure 1*b*). The uninduced LyD9 cells were negative for surface IgM, but 17% of the induced LyD9 cells were stained with anti-IgM (figure 1*c*). We used the bone marrow stromal cells of NZB mice, which have *n* allotype IgM molcules, so that IgM-bearing cells of LyD9 (*j* allotype) could be distinguished from those of NZB mice (*n* allotype) with use of the MB86 monoclonal antibody (Nishikawa *et al.* 1986). IgM-bearing cells derived from NZB mice were few (at most 4%) (figure 1*d*), showing that LyD9 cells differentiated *in vitro* with a high frequency. The number of cells producing IgM increased greatly when LyD9 cells were treated beforehand with 5-azacytidine (Kinashi *et al.* 1988).

We then stained the induced LyD9 with anti-IgG antibody to see whether or not these cells also underwent class switch to produce IgG antibody. About 10% of LyD9 cells were IgG-positive, whereas IgG-bearing cells were not found in the control culture without LyD9 cells.

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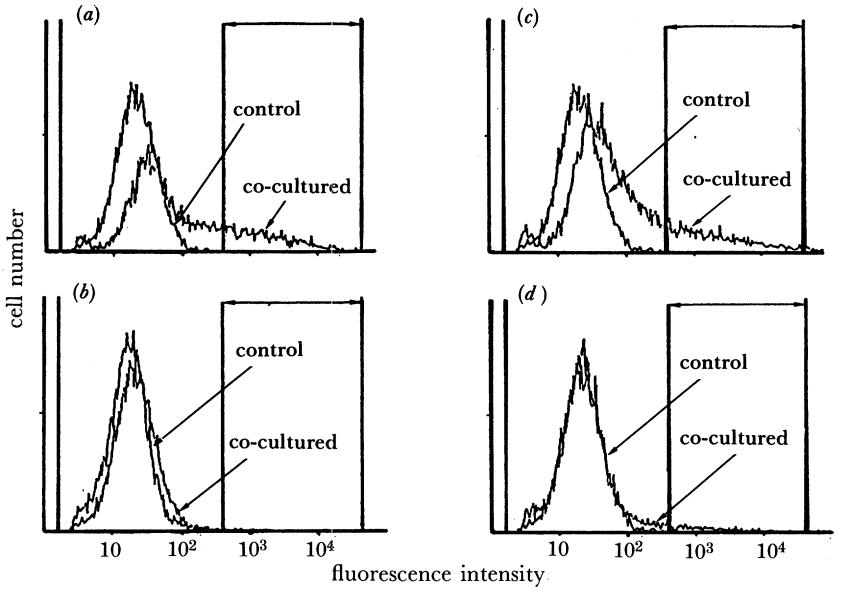

FIGURE 1. Induction of the surface IgM on LyD9 cells by the co-culture with bone marrow accessory cells. LyD9 cells were co-cultured with bone marrow accessory cells. After 10 days of culture, cells were harvested for immunofluorescence staining with anti-B220 antibody (*a*), anti-Thy-1.2 antibody (*b*), polyclonal anti-murine IgM antibody (*c*), or MB86 monoclonal anti-μ chain antibody (*d*). The anti-μ chain antibody used in (*c*) does not distinguish *j* and *n* allotypes, whereas the one used in (*d*) recognizes the *n* allotype but not *j* allotype. Uninduced LyD9 cells were used as negative controls. Stained cells were analysed by a fluorescence-activated cell sorter (ABCAS100). Taken from Kinashi *et al.* (1988).

TABLE 1. PRODUCTION OF IgM AND IgG BY LyD9 CELLS CO-CULTURED WITH BONE MARROW STROMA CELLS

(Culture supernatants of 10-day co-culture described in Materials and methods were assayed for immunoglobulin by ELISA.)

LyD9	IgM/(ng ml^{-1})	IgG/(ng ml^{-1})
+	3200	1400
−	< 1	< 8

The production of IgM and IgG was further confirmed by measuring the amounts of IgM and IgG in the supernatant of the co-culture (table 1). After a 10-day co-culture with stromal cells, 3.2 μg ml^{-1} of IgM and 1.4 μg ml^{-1} of IgG were secreted by LyD9 cells. Negligible amounts of immunoglobulin were found in supernatants of stromal cells without LyD9 cells.

To confirm the expression of immunoglobulin by induced LyD9 cells, we measured immunoglobulin mRNA in LyD9 cells co-cultured with bone marrow stromal cells. RNA blot hybridization showed that the C_μ and C_κ transcripts in differentiated LyD9 were full-length mRNAs and not sterile transcripts of the IgC genes (Kinashi *et al.* 1988).

Random V–D–J recombination

We also tested whether the IgM-bearing cells had rearranged selected V_H and V_κ segments of the immunoglobulin gene. Southern blot filters of digested DNA from induced LyD9 cells that were 15% IgM positive were hybridized with J_H or J_κ probes and also with the C_κ probe as an internal reference (figure 2*a*, lanes 1–4). We did not find any discrete rearranged bands hybridized with either the J_H or J_κ probe. After longer exposure (figure 2*b*, lanes 1–4) smears

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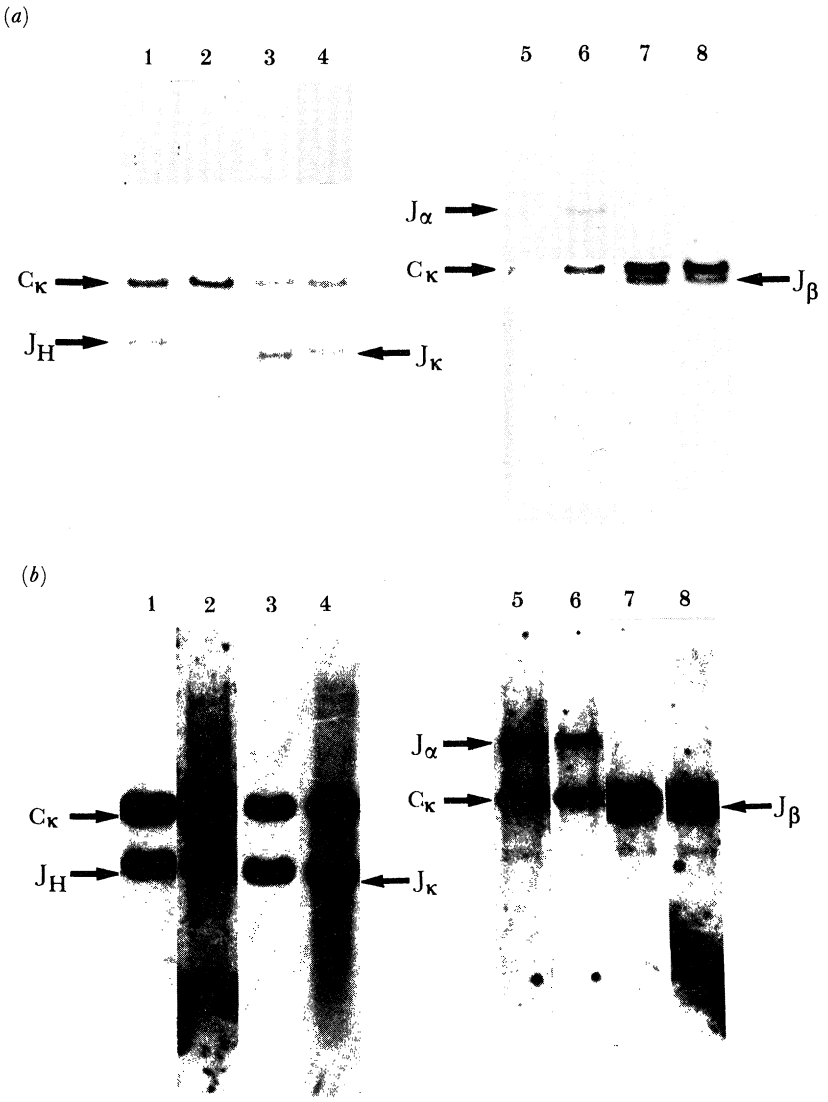

FIGURE 2. Southern blot analysis of immunoglobulin gene loci and T-cell receptor loci in DNA of induced LyD9 cells. DNA was extracted from LyD9 cells co-cultured with bone marrow stromal cells for nine days, 15% of which expressed IgM on their surface, and was digested with *Xba*I. As the *Xba*I site is between the J_κ and C_κ genes, the size of the C_κ fragments should not be changed by the immunoglobulin gene recombination. Uninduced LyD9 DNAs were used as controls. Filters were exposed briefly to quantitate relative intensity of bands (*a*) and longer to see whether smeary bands appeared (*b*). Origins of DNA used are: (i) uninduced LyD9 in lanes 1, 3, 5, and 7, and (ii) induced LyD9 in lanes 2, 4, 6, and 8. Probes used are: (i) J_H and C_κ (internal control) probes in lanes 1 and 2; (ii) J_κ and C_κ probes in lanes 3 and 4; (iii) J_α and C_κ probes in lanes 5 and 6, and (iv) J_β and C_κ probes in lanes 7 and 8. The germ line *XbaI* fragments of the C_κ (5.8 kilobases (kb)), J_H (3.8 kb), J_κ (3.4 kb), J_α (9.0 kb), and J_β (5.3 kb) probes are shown by arrows. Taken from Kinashi *et al.* (1988).

of both larger and smaller sizes appeared, and the germ line J_H or J_κ bands had reduced intensities. The intensity of the germ line J_H and J_κ bands relative to that of the C_κ band decreased to 61% and 65%, respectively, at day nine of co-culture when compared with that in uninduced LyD9 cells.

These results show that the J_H and J_κ segments were associated with heterogenous V_H and V_κ segments to yield J_H and J_κ fragments of different lengths. The resuls excluded the possibility

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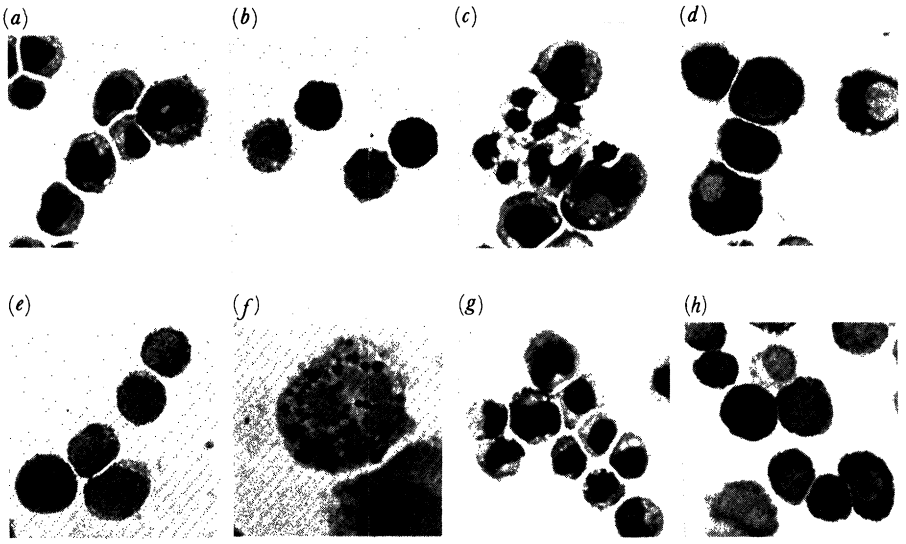

FIGURE 3. Morphological and cytochemical analyses of the LyD9 clone and its derivatives. Cytocentrifuge preparations of induced LyD9 (*a*, *b*), LS-1 (*c*–*f*), induced K-4 (*g*, *h*) and were stained with May–Grünwald–Giemsa (*a*, *c*, *g*), for α-naphthyl-butyrate esterase (*b*, *d*, *h*), and for chloroacetate esterase (*e*) and for peroxidase (*f*) as described (Goud *et al.* 1975). The magnification is ×1000 in (*f*) and ×400 in the others. LS-1, LyD9 and K-4 cells were grown in media containing GM-CSF, IL-3 and IL-4, respectively.

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that the co-culture procedure selected pre-existing IgM-bearing clones in LyD9 cells. No rearrangement at T-cell receptor loci occurred in induced LyD9 as the relative intensities of the germ line J_α and J_β segments were unchanged (figure 2*a*, lanes 5–8); no significant smears appeared, even after long exposure (figure 2*b*, lanes 5–8). No rearrangement of the J_γ segment was found in induced LyD9 cells (data not shown).

Differentiation of the LyD9 clone into myeloid cells as well as B lymphocytes

Morphological and cytochemical studies on induced LyD9 cells showed that induced LyD9 cells contained not only lymphoid cells but also myeloid cells (figure 3*a*, *b*, plate 1). Some of the induced LyD9 cells contained non-specific esterase activity. Peroxidase-staining was weak but significant (data not shown). The frequency of myeloid cells was roughly proportional to the frequency of Mac-1^+ cells (26%) determined by the FACS analysis (Kinashi *et al.* 1989). The culture of stromal cells without the LyD9 cells, however, did not give rise to either lymphoid or myeloid cells.

Establishment of GM-CSF-dependent clones from induced LyD9 cells

Induced LyD9 cells harvested after 10 days of the co-culture with stromal cells were transferred onto a stromal cell line ST-2, known to support both B lymphopoiesis and myelopoiesis from bone marrow progenitor cells (Ogawa *et al.* 1988). Stromal cell (ST-2)-dependent clones were established from this culture. Uninduced LyD9 cells could not survive on the ST-2 cell and died within a week. One of such clauses, LS-1 was studied extensively.

GM-CSF-dependent clones were also established from induced LyD9 cells by repeated cloning in GM-CSF. One of these clones, called K-GM, had a surface phenotype similar to that of the LS-1 clone (Mac1^+, B220$^-$ and IgM$^-$). The surface profiles of LS-1 and K-GM are consistent with those of myelid lineage cells. Southern blot hybridization showed that both LS-1 and K-GM contained the same integration profile of the *Neo*^{r} gene as the parental LyD9 clone.

LS-1 proliferated well in the presence of either IL-3, IL-4, GM-CSF, M-CSF or G-CSF without ST-2 cells. However, neither IL-1, IL-2, IL-5 or IL-6 could support the growth of Ls-1 (table 2). In fact, the LS-1 cells were also maintained in GM-CSF. K-GM grew not only in GM-CSF, but also in IL-3, and to a lesser extent in IL-4.

Morphological evidence that LS-1 and K-GM are myeloid lineage cells

The LS-1 cells cultured in the presence of G-CSF and M-CSF contained cells similar to neutrophils and macrophages, respectively. Both macrophage- and neutrophil-like cells appeared in the LS-1 cells cultured in the presence of GM-CSF (figure 3 *c*–*f*). The LS-1 cells expressed high levels of non-specific esterases. Peroxidase-containing granules were found in neutrophil-like cells of the LS-1 cells, and also in macrophage-like cells, albeit in lesser amounts. The K-GM cells contained both macrophage- and granulocyte-like cells that expressed non-specific esterases and peroxidase (data not shown). Typical macrophages and granulocytes appeared more frequently in the LS-1 cells and in the K-GM cells grown in soft agar cultures than in suspension cultures. Furthermore, PMA-treated LS-1 cells showed a strong non-specific phagocytosis activity. These results show that the LS-1 and K-GM clones belong to the granulocyte–macrophage lineage.

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Table 2. Response of LyD9 and its derivatives to growth factors

Cells (4×10^4) were incubated in microplate wells with the complete medium (200 µl) containing growth factors. The concentration of each growth factor was chosen so that the maximal plateau level of proliferative response was obtained. [^{3}H]thymidine (0.5 µCi) was added to each culture well during the last 6 h of the 48-hour incubation. Radioactivities incorporated into cells were measured by filtration and acid washing. The data are the mean of triplicate plates. The standard error was less than 10% of the mean throughout samples. Purified recombinant (r) human IL-lα and IL-2 were provided by Dainippon Pharmaceutical Company and Takeda Pharmaceutical Company (Japan), respectively. Purified murine rIL-3 was provided by DNAX Research Institute. Mouse rIL-4 and rIL-5 were obtained from X63 Ag8 myeloma cells transfected with IL-4 and IL-5 cDNAs. Purified human rIL-6 and murine recombinant GM-CSF were provided by Dr T. Hirano (Osaka University) and Dr T. Sudo (Toray Basic Research Institute), respectively. Purified rG-CSF and human rM-CSF were provided by Dr S. Nagata (Osaka Bioscience Institute).

growth factors added	[^{3}H]thymidine (c.p.m.) incorporation into cells			
	LyD9	K-4	LS-1	K-GM
medium	355	276	805	245
IL-1	422	461	796	827
IL-2	656	551	591	433
IL-3	26542	30291	35528	20051
IL-4	1451	31456	18349	4107
IL-5	316	1014	6217	310
IL-6	221	1120	1011	521
GM-CSF	195	1394	45904	36702
M-CSF	210	937	27155	242
G-CSF	154	827	31648	711

Table 3. Effects of antibodies on differentiation of LyD9 cells by co-culture with bone marrow stroma cells

These antibodies in hybridoma culture supernatant were added to the co-culture in a 1:9 (mass by volume) (10% final volume), which did not affect proliferation of LyD9 in the presence of IL-3. Sources of antibodies are cited as references in parentheses.

antibodies added	cells in seven-day co-culture number $\times 10^{-3}$ per well	IgM$^+$(%)
anti-IL-4	3.3	0
anti-LFA-1	5.4	< 2.5
anti-B220	28.3	9.0
anti-Mac-1	19.3	6.1
none	23.7	8.2

Involvement of IL-4 and LFA-1

Although neither IL-4 nor IL-5 supported differentiation of LyD9 cells into B cells, the addition of an anti-IL-4 antibody blocked proliferation and differentiation of LyD9 cells in co-culture with stroma cells (table 3). It is likely that proliferation and differentiation of LyD9 cells requires some unknown growth factor or factors in addition to IL-4. An anti-IL-5 antibody did not affect differentiation of LyD9 cells (data not shown). The addition of an anti-LFA-1 antibody strongly inhibited proliferation and differentiation of LyD9 cells. LFA-1 belongs to the integrin family and contributes to cell–cell adhesion by interaction with the intercellular adhesion molecule-1 (ICAM-1). LyD9 and stromal cells may require direct contact with each other. LyD9 cells were seen to have grown with direct contact with stromal cells when the cells were observed microscopically. It is worth noting that this amount of antibody did not affect proliferation of LyD9 cells in the presence of IL-3. Antibodies against other surface markers, such as B220 or Mac-1, did not affect differentiation and proliferation

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significantly. When these results are considered together, they suggest that LyD9 cells may be able to receive growth factors only when they have direct contact with stromal cells because the factor is in low concentration or because direct contact of cells stimulates production of these factors or their receptors.

Establishment of IL-4-dependent clones from induced LyD9 cells

We attempted to isolate a possible intermediate cell between LyD9 and B cells by culturing induced LyD9 cells with IL-4. Several IL-4-dependent clones were established by repeated cloning of induced LyD9 cells in the presence of IL-4. Surface phenotypes of one of these clones, K-4, were almost identical to those of LyD9 (Palacios *et al.* 1987) except that expression of the B220 antigen was about twofold augmented. The K-4 clone was certainly derived from the LyD9 clone because K-4 had the same integration pattern of the *Neo*^{r} gene as the parental LyD9 clone used.

We compared the growth-response profile of the parental LyD9 and K-4 clones with various recombinant soluble factors (IL-1–6, granulocyte–macrophage (GM)-colony stimulating factor (CSF), M-CSF and G-CSF) (table 2). LyD9 cells grew in IL-3, and responsed to Il-4, albeit slightly and transiently. On the other hand, K-4 cells were maintained in medium containing either IL-4 or IL-3, and responded to IL-4 as strongly as IL-3. K-4 did not proliferate in IL-5, IL-6 or GM-CSF although weak responses to these factors were observed.

Differentiation of the K-4 clone into myeloid as well as B lymphocytes

We examined whether the differentiation capacity into myeloid cells as well as B cells is maintained by the K-4 clone. K-4 cells were subjected to surface staining after co-culture with bone marrow stromal cells under conditions known to promote B-cell differentiation. The proportions of induced K-4 cells that strongly expressed the IgM, B220 and Mac-1 antigens were similar (13%, 22%, 26%, respectively) to those for the induced LyD9 clone. Morphological studies identified myeloid as well as lymphoid cells in induced K-4 cells (figure 3*g*, *h*). Furthermore, GM–CSF-dependent cell lines similar to the K-GM clone were obtained by culturing induced K-4 cells in GM-CSF. These results indicate that the K-4 clone can differentiate into myeloid cells as well as B lymphocytes when co-cultured with bone marrow stromal cells. In contrast, the LS-1 and K-GM clones did not ever differentiate into B lymphocytes in the same co-culture system. The results led us to conclude that the LS-1 and K-GM clones had been committed to the myeloid lineage. These results, together with the fact that the anti-IL-4 monoclonal antibody blocked the differentiation of the LyD9 clone, enabled us to conclude that the K-4 clone is an intermediate in the differentiation pathway of the LyD9 cell into myeloid cells as well as B cells.

Discussion

We have shown that the IL-3-dependent progenitor clone (LyD9) and its IL-4-dependent derivative (K-4) have capacities to differentiate into myeloid as well as B lymphocytes when co-cultured with bone marrow stromal cells. The induced cells gave rise to long-term stromal or CSF-dependent cell clones that had differentiated into the granulocyte–macrophage lineage. Available surface markers could not distinguish between the LyD9 and K-4 clones except for the increased B220 expression on the K-4 clone. Studies on phenotypic changes in the intermediate clone K-4 other than the growth factor requirement will provide a clue to

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understand molecular events required for differentiation of early precursor cells in the bone marrow.

It was interesting that IL-4 was necessary during the differentiation of LyD9 cells into B cells and granulocyte–macrophage cells because IL-4 is known to be a soluble factor that acts on T cells, B cells and mast cells (Noma *et al.* 1986; Sideras *et al.* 1988). Because of both multipotential K-4 cells and granulocyte–macrophage progenitor cells, LS-1 was able to respond to IL-4; IL-4 would play an important role in differentiation of haematopoietic progenitor cells in agreement with the report that IL-4 enhances the proliferation of CFU-GM, CFU-e and CFU-mix in the presence of appropriate factors (Peschel *et al.* 1987). Although IL-4 is involved in an early phase of differentiation, IL-4 alone cannot induce differentiation of the LyD9 cell. It remains to be determined whether LyD9 cells or stromal cells produce IL-4 during the co-culture. Stromal cells seem to provide differentiation signals to LyD9 and K-4 cells during their physical contact.

Several investigators (Davidson *et al.* 1988; Greaves *et al.* 1986) have described leukaemic cells or haematopoietic cells transformed with oncogenes that express both B-cell and myeloid characters. These studies suggest the existence of a common B-cell–myeloid progenitor cell in agreement with this study. Some other leukaemic cells have properties of both the T-lymphocyte and myeloid lineages. It is worth noting that both LyD9 cells (Palacios *et al.* 1987) and HAFTL-1 cells (Peschel *et al.* 1987) express the Ly-1 surface antigen. Ly-1$^-$ IL-3-dependent pro-B lymphocyte clones were shown to give rise to B lymphocytes *in vivo*, but not to lymphocytes or myeloid cells (Palacios & Steinmetz 1985).

The second point suggested from the present studies is that acquisition of capacities to respond to different growth factors is associated with differentiation of the LyD9 clone into B lymphocytes and myeloid cells. It will be interesting to determine whether the change of growth factor dependence is induced either by increased numbers of specific receptors or by induction of the intracellular signal transduction machinery specific to receptors. We will be able to test whether expression of a specific receptor can induce differentiation into a particular linage when cDNAs of various receptors are available.

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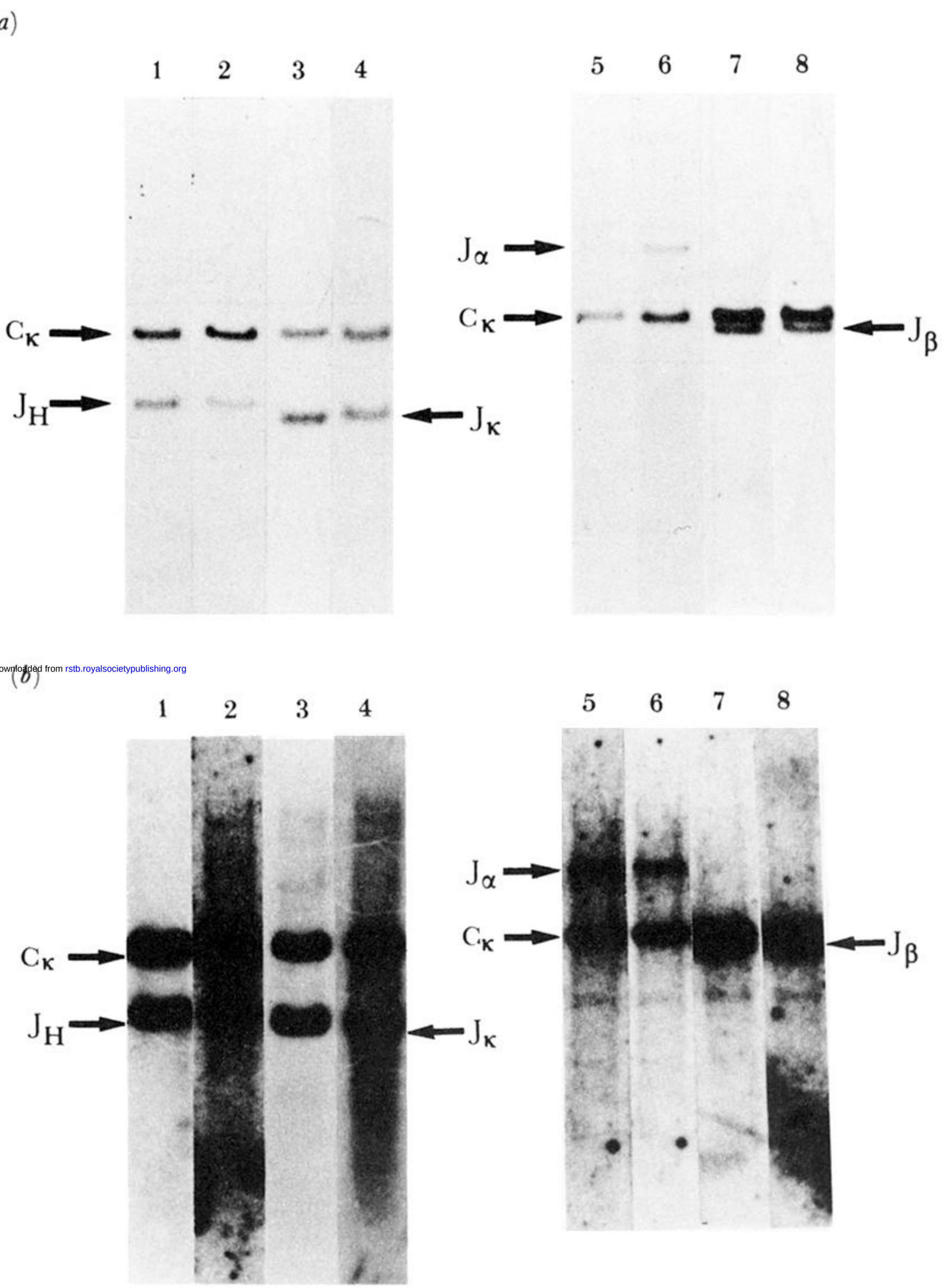
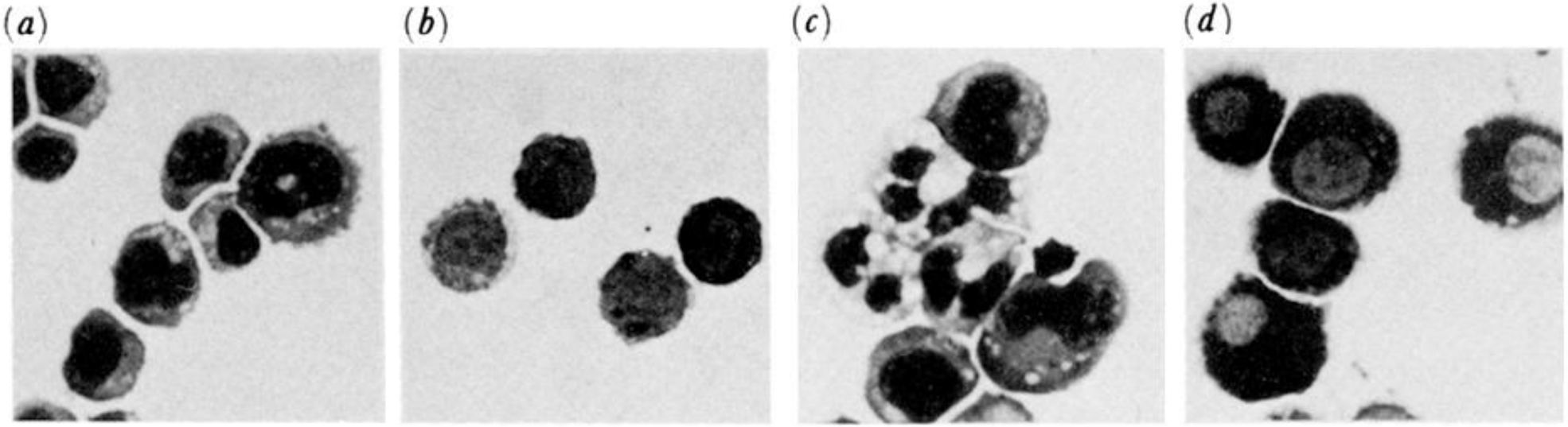


FIGURE 2. Southern blot analysis of immunoglobulin gene loci and T-cell receptor loci in DNA of induced LyD9 cells. DNA was extracted from LyD9 cells co-cultured with bone marrow stromal cells for nine days, 15% of which expressed IgM on their surface, and was digested with *Xba*I. As the *Xba*I site is between the J_κ and C_κ genes, the size of the C_κ fragments should not be changed by the immunoglobulin gene recombination. Uninduced LyD9 DNAs were used as controls. Filters were exposed briefly to quantitate relative intensity of bands (*a*) and longer to see whether smeary bands appeared (*b*). Origins of DNA used are: (i) uninduced LyD9 in lanes 1, 3, 5, and 7, and (ii) induced LyD9 in lanes 2, 4, 6, and 8. Probes used are: (i) J_H and C_κ (internal control) probes in lanes 1 and 2; (ii) J_κ and C_κ probes in lanes 3 and 4; (iii) J_α and C_κ probes in lanes 5 and 6, and (iv) J_β and C_κ probes in lanes 7 and 8. The germ line *XbaI* fragments of the C_κ (5.8 kilobases (kb)), J_H (3.8 kb), J_κ (3.4 kb), J_α (9.0 kb), and J_β (5.3 kb) probes are shown by arrows. Taken from Kinashi *et al.* (1988).



GURE 3. Morphological and cytochemical analyses of the LyD9 clone and its derivatives. Cytocentrifuge preparations of induced LyD9 (*a*, *b*), LS-1 (*c*–*f*), induced K-4 (*g*, *h*) and were stained with May–Grünwald–Giemsa (*a*, *c*, *g*), for α-naphthyl-butyrate esterase (*b*, *d*, *h*), and for chloroacetate esterase (*e*) and for peroxidase (*f*) as described (Goud *et al.* 1975). The magnification is ×1000 in (*f*) and ×400 in the others. LS-1, LyD9 and K-4 cells were grown in media containing GM-CSF, IL-3 and IL-4, respectively.

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