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Immortalization of Murine Leukocytes by Oncogenes. I. Attempts to Immortalize Splenic Dendritic Cells Resulted in the Production of Nondendritic Transformant Cells

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Abstract Murine lymphoid dendritic cells (DC) were purified from spleen cells and transfected with oncogenic retroviral vectors expressing either v-*src* or activated c-Ha-*ras* oncogene in a helper virus-free system. The transfectants were cultured in medium alone or in combination with tumor promoters, phorbol ester and/or calcium ionophore. Although both oncogenic retroviruses induced nondendritic transformants, they did not induce DC transformants even in combination with the tumor promoters, indicating that DC were very resistant to the action of these oncogenic retroviruses *in vitro*.

Introduction

The establishment of the method to purify dendritic cells (DC) from mouse spleens made it possible to verify functional roles of DC in immune responses. Extensive studies conducted by several research groups including ours have revealed that DC are principal accessory cells in the initiation of immune responses (Muramatsu, 1985; Steinman & Nussenzweig, 1980; Steinman et al., 1986). DC, which are nonphagocytic cells, constitutively express class II MHC antigens, form clusters with T cells, and activate them (Inaba & Steinman, 1986). However, biochemical analysis of accessory function of DC is hampered by the difficulties to prepare enough quantities of purified DC. It is not clear whether lymphocyte-activating molecules are present on the surface of DC or whether such molecules are secreted by DC during cluster formation with lymphocytes. Although DC are bone marrow-derived cells (Steinman et al., 1974), their differentiation pathway is not elucidated. It is also unknown whether they are end-cells or still retain an ability to proliferate in response to appropriate growth factors or to some stimulations like lymphocytes and macrophages do.

Abbreviations used: DC, dendritic cells; LTR, long terminal repeat; Mϕ, macrophages; MHC, major histocompatibility complex; PMA, phorbol 12-myristate 13-acetate.

This study was performed to examine whether oncogenic retroviruses had an ability to immortalize splenic DC. Recombinant retroviruses containing *src* or *ras* oncogene succeeded in immortalizing nondendritic leukocytes which were present in a small quantity in DC-enriched preparations. However, under the present experimental conditions, these viruses did not induce DC transformants even in the presence of tumor promoters, phorbol ester and calcium ionophore.

Materials and Methods

Oncogenic vectors and virus-producing cell lines. pZipNeoSrc (Fig. 1) was constructed by insertion of the 3.1–kb *Eco*RI fragment encoding v-*src* (Takeya et al., 1981) into the *Bam*HI site of pZipNeoSV(X)1 (Cepko et al., 1984) with *Bam*HI linkers (Takara Shuzo Co., Ltd., Kyoto). This vector contains Moloney murine leukemia virus (Mo-MuLV) long terminal repeats (LTR) and expresses the Tn5 *neo* gene through a subgenomic mRNA from the 5' viral LTR. The *neo* gene conveys resistance to kanamycin in *E. coli*, and to G418 in mammalian cells. pZipNeoRas (Fig. 1) was

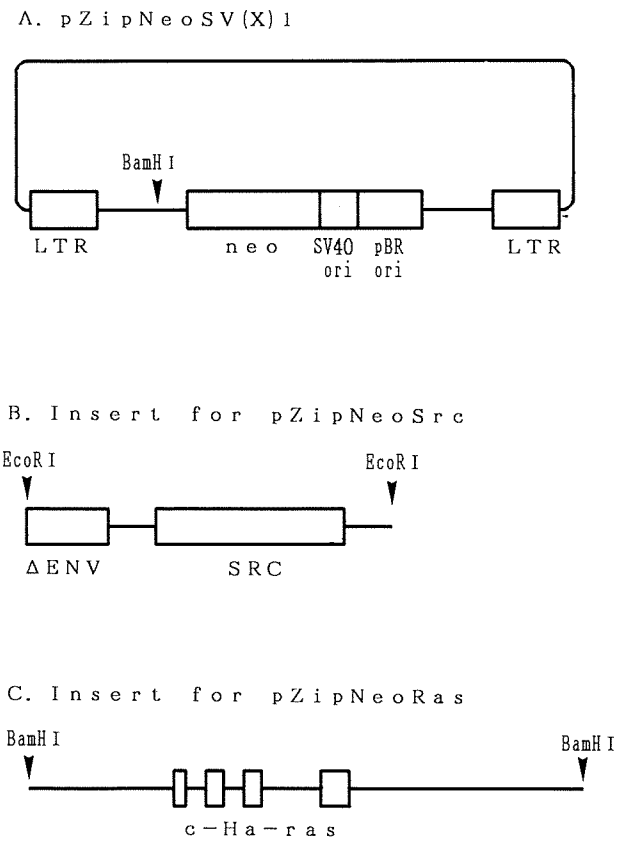

Fig. 1. Structure of pZipNeoSV(X)1 and inserts for pZipNeoSrc and pZipNeoRas. A. pZipNeoSV(X)1 plasmid (10.1 kb). *BamH*I site to insert oncogenes is indicated by an arrow head. B. The 3.1-kb *Eco*RI fragment of pTT107 used to construct pZipNeoSrc. C. The 6.4-kb *Bam*HI fragment of pT24 used to construct pZipNeoRas.

constructed by insertion of the 6.4-kb *Bam*HI fragment encoding activated c-Ha-*ras* (Goldfarb et al., 1982) into the *Bam*HI site of pZipNeoSV(X)1.

Retrovirus-producing cell lines, ψNeoSrc and ψNeoRas, were established from ψ2 cell line (Mann et al., 1983) by transfection with pZipNeoSrc and pZipNeoRas, respectively, using calcium phosphate method (Mori et al., 1987), and by selection of G418 (Geniticin: Sigma Chemical Company, St. Louis, MO) resistant colonies. Virus-producing cell lines were cultured in fresh medium containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) under a subconfluent culture condition for 24 hr. After centrifugation at 1500 g for 10 min and membrane filtration (ϕ =0.45 μm; Millipore Corporation, Bedford, MA), cell-free culture supernatants containing viruses were used immediately for transfection experiments.

Mice. Male and female mice of a strain, (BALB/c×DBA/2)F_1 (CD2F_1; Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka) were used at the age of 2–4 months.

Dendritic cells. DC were prepared from spleen cells as described previously (Komatsubara et al., 1986). Briefly, spleen cells suspended in dense bovine serum albumin (Armour Pharmaceutical Co., Kankakee, IL) solution (ρ =1.082) were centrifuged at 1500×g for 25 min, and floating cells (low density cells) were cultured in plastic dishes for 2–3 hr. After removing nonadherent cells, adherent cells (low density adherent cells) were cultured for an additional 18–24 hr. The cells which became nonadherent during the culture period were collected (DC-enriched preparations), and incubated in a new dish for 1–2 hr to remove contaminating adherent cells. Nonadherent cells were then collected by gentle swirling the dish and aspiration, and used as purified DC preparations. The constituents of DC-enriched and -purified preparations were as follows: DC, 70–80% and about 90%; B lineage cells (B220-positive cells), about 10% and 6-8%; Mϕ, 1–3% and<1%; T cells, 1–3% and<1%; fibroblasts,< 0.1% and<0.1%.

Virus infections. DC-enriched, DC-purified, or low density adherent cell preparations were infected with oncogenic retroviruses and cultured according to the schedules shown in Fig. 2. In some experiments, viral infections were performed in the presence of 8 μg/ml of polybrene (Sigma) to facilitate infectivity. In schedule III, DC were purified from low density adherent cells after viral infection, and cultured in the presence of IL-3 (WEHI-3 culture supernatants), 1 or 5 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma), 100 ng/ml of calcium ionophore (A23187; Sigma), or PMA plus calcium ionophore.

Culture medium. Unless otherwise mentioned, cells were cultured in Dulbecco's modified Eagle medium (DMEM; Nissui Seiyaku Co., Tokyo) supplemented with 5-10% FBS and antibiotics. In order to select transformants, 400 μg/ml of G418 was usually added to this medium.

Growth in soft agar. Anchorage-independence of transformants was examined using SeaPlaque agarose (FMC Corp., Rockland, ME). Ten thousands cells in culture medium containing 0.4% agarose were layered over 0.6% agarose medium in dish, and cultured for several days. Colony-formation in soft agarose medium was observed by inverted phase-contrast microscopy.

Nonspecific esterase. Nonspecific esterase activity of glutaraldehyde-fixed cytocentrifuged cell preparation were examined using α-naphthylacetate as a substrate.

Peritoneal macrophages (Mϕ) and a nonspecific esterase-positive cell line, WEHI-3, were examined as positive controls.

Results

Transfection of DC-enriched preparations with oncogenic retroviruses. Purified DC in culture are short-lived; they die out in several days. When we culture crude DC preparations such as low density adherent cells, some of DC may survive for a week more. However, such crude preparations contain fibroblasts and Mϕ which grow during the culture period and dominate the culture soon. Therefore, to reduce the content of these cell types, we first used DC-enriched preparations and infected them with recombinant retroviruses which expressed either v-*src* or activated c-Ha-*ras* oncogene (Fig. 2, schedule I). In two of four independent experiments, rapidly growing transformants emerged within a week after transfection. They were, however, round nonadherent cells and did not look like dendritic cell lines by morphology. DC

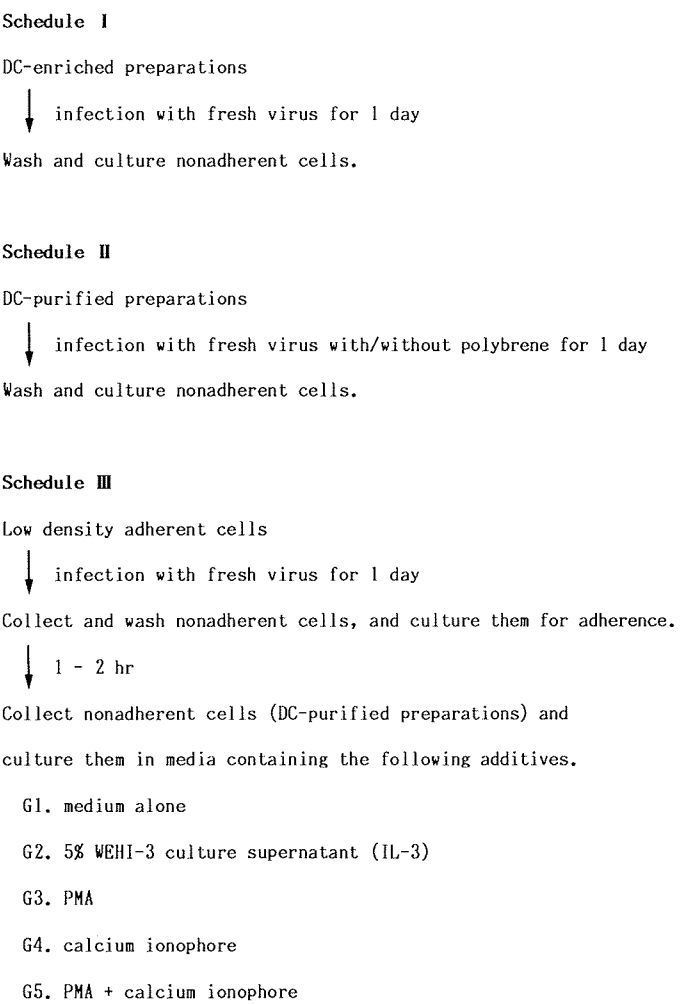

Fig. 2. Schedules used in this study to examine transformation of DC.

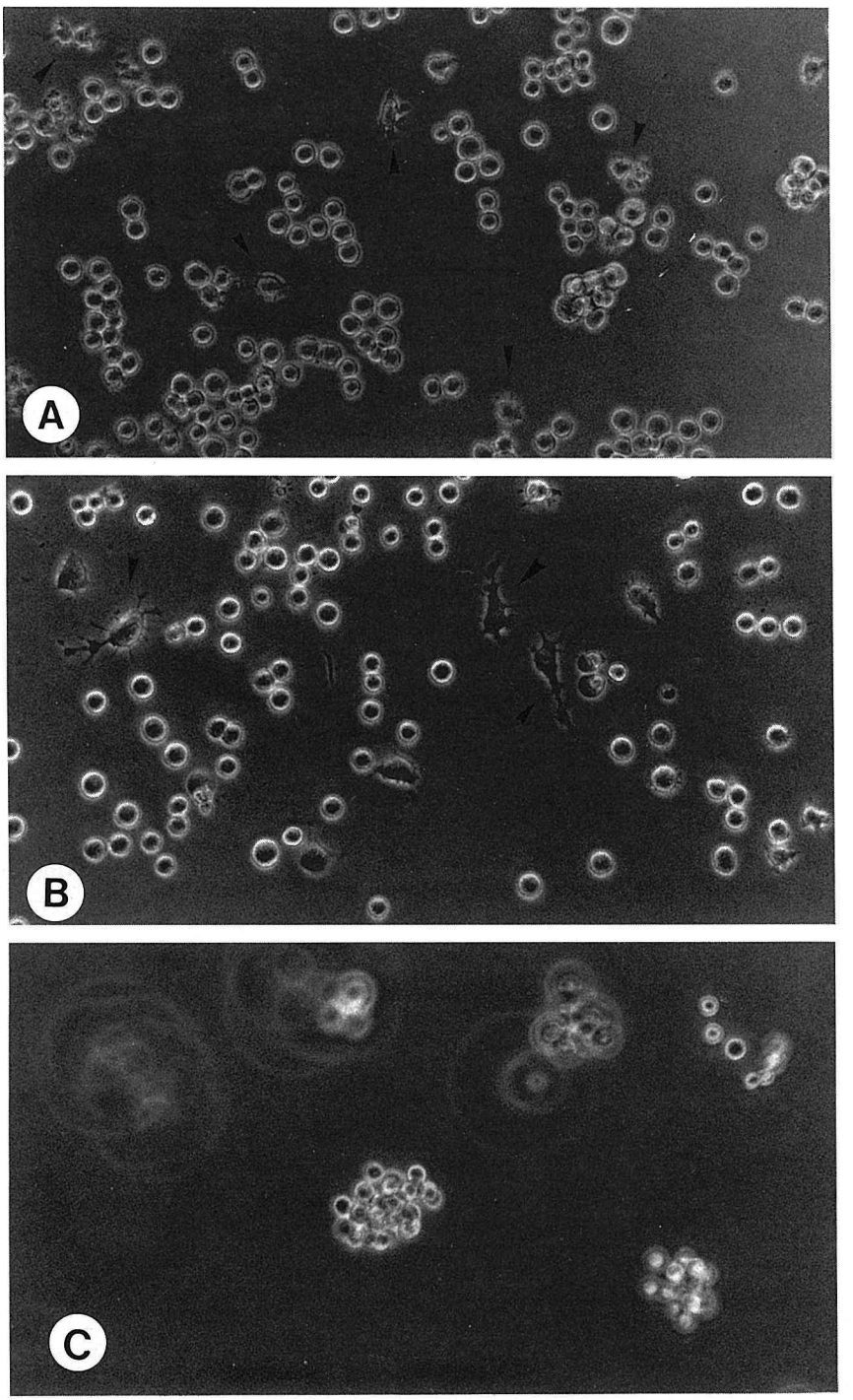

Fig. 3. Microscopic observation of the transformant cell lines in culture. A. RA1 cell line were cultured in the medium containing 5% FBS. Arrowheads indicate the irregularly shaped cells. B. RA1 cell line were cultured in the serum-free medium for 2 days. Arrowheads indicate the cells of apparent dendritic morphology. C. Colonies formed after 2-day culture of SR1 cell line in 0.4% soft agarose medium.

died out in a week, and fibroblasts and Mϕ dominated the culture in several weeks. Uninfected DC preparations did not raise any transformants.

Transfection of DC-purified preparations. Since DC-enriched preparations gave rise to nondendritic transformants which soon overpopulated the cultures, we next used DC-purified preparations for transfection experiments (Fig. 2, schedule II). We did four independent experiments, but in none of the experiments emerged DC transformants. DC died out in 2–4 days. Culturing DC with feeder cells such as splenic adherent cells extended the survival of DC for several days more, but was ineffective for production of DC transformants.

Effect of tumor promoters. In order to examine the effect of tumor promoters on the transforming activity of v-*src* and Ha-*ras* oncogenes, we performed an experiment shown in Fig. 2, schedule III. Low density adherent cells were infected with oncogenic retroviruses for 1 day. DC were purified thereafter and cultured in the presence of PMA and/or calcium ionophore. DC in medium alone or in the presence of calcium ionophore died out in 2–4 days. DC cultured with PMA or PMA plus calcium ionophore enlarged their cell size, and some of them, especially in the presence of PMA plus calcium ionophore, survived for 1–2 weeks. However, they did not initiate proliferation.

Characterization of round nonadherent transformants. As mentioned in the first section of results, we have established nondendritic transformant cell lines from DC-enriched preparations. The majority of cells in each cell line was rapidly growing, round, and nonadherent (Fig. 3A). Some of cells in the cell lines, however, were adherent and/or irregularly shaped, and some of them showed more evident dendritic morphology after the short-term culture in serum-free medium (Fig. 3B). Thus, the transformant cell lines might be a mixutre of several cell types, or of cells at different differentiation stages. They proliferated in medium containing up to 800 μg/ml of G418, and were able to grow in soft agarose medium in an anchorage-independent manner (Fig. 3C). They were negative for nonspecific esterase activity, a marker for

Table 1. Characterization of *src*(SR)- and *ras*(RA)-transformant cell lines.

Cell line	Cell growth		Immunoglobulin		Nonspecific esterase[5]
	in vivo[1]	in soft agar (%)[2]	surface[3]	cytoplasmic[4]	
SR1	+	+ (34)	−	−	−
SR2	+	+ (41)	−	−	−
SR3	+	+ (49)	−	−	−
SR4	+	+ (46)	−	−	−
RA1	+	+ (35)	−	−	−
RA2	+	+ (65)	−	−	−

[1] Transformant cell lines (5×10^6) were intraperitoneally injected in 4-wk old syngeneic CDF_1 mice.
[2] Colony-forming activity (and % colony-forming efficiency) of 10^2 transformant cell lines which were cultured in 0.4% agarose-containing semisolid medium.
[3] Cells were stained with FITC-conjugated goat anti-mouse immunoglobulins.
[4] Acetone-fixed cell preparations were stained with FITC-conjugated goat anti-mouse immunoglobulins.
[5] Examined by an α-naphthylacetate method.

macrophages. Table 1 summarizes some of the typical characteristics of these cell lines.

Discussion

In order to attempt production of DC transformants, we used a retroviral system, in which pZipNeoSV(X)1 vector (Cepko et al., 1984) and $\psi 2$ cell line (Mann et al., 1983) were utilized. This system is very sophisticated in that transformed cell lines manifest few side effects due to the vector system, and are expected to maintain normal physiological traits fairly well. We constructed pZipNeoSrc and pZipNeoRas oncogenic retroviral vector which contained v-*src* and activated c-Ha-*ras* oncogenes, respectively, but not other retroviral genomic components such as *gag*, *pol*, and *env* gene. As to viral packaging, we used $\psi 2$ cell line. This cell line expresses all Mo-MuLV gene products but can not produce infectious viruses due to the genomic defect of ψ sequence, required for encapsidation of the viral genome. By transfecting pZipNeoSrc and pZipNeoRas vectors into $\psi 2$ cell line, we established ψNeoSrc and ψNeoRas cell lines, respectively. Both cell lines produced oncogenic retroviruses which easily transformed Rat–2 fibroblastic cell line and conveyed G418 resistance.

This study was done in order to establish DC lines, but all the transformants, which we obtained from DC-enriched cell preparations, were nondendritic cells. Splenic fibroblasts had an ability to proliferate *in vitro*. After transfection with oncogenic retroviruses, some of them were transformed during *in vitro* passage to form typical transformation foci (our unpublished observation). On rare occasion, macrophage-like cell lines also emerged (unpublished observation). All the cell lines mentioned above, however, were not characterized any further. Another type of established transformants was round nonadherent cells. They emerged from DC-enriched preparations and seemed nondendritic cells, though some of them were adherent and/or irregularly shaped. We will characterize these transformants in detail elsewhere (Komatsubara et al., 1988).

Although both oncogenic retroviruses used in this study were able to induce transformants of several cell types, they did not succeed in producing DC transformants. Most probable reasons for this are as follows. The integration of retroviral genomes into target cell chromosomes requires, or at least is greatly facilitated by, cell growth of target cells. Since purified DC did not proliferate, the integration of retroviral vectors into DC chromosomes might take place in too low frequency to detect transformation. Another possible reason is ascribed to the short life of purified DC *in vitro*. They were able to survive only for several days. Therefore, even if DC were transfected with retroviruses, there might not be enough time to lead the DC into a transformed stage. It may be also possible that DC have a rather higher level of anti-oncogene activity which counteracts the action of the transfected oncogenes and keeps DC in normal phenotype. Alternatively, the failure may be simply due to the lack or shortage of the appropriate receptor for Mo-MuLV on the surface of DC, resulting in too low viral infectivity to induce transformation.

Since purified DC alone were difficult to be maintained *in vitro*, we tried to extend their survival to facilitate transformation. First, transfected DC were cultured with splenic feeder cells, by which their survival was extended for an additional several

days. Second, transfected DC were cultured in the presence of tumor promotors, PMA and/or calcium ionophore. PMA, especially in combination with calcium ionophore, induced the increase of cell size of DC, and some of DC survived for 1–2 weeks without initiating proliferation. These attempts, however, were ineffectual to induce DC transformants.

There are several possible ways not tested in this study. It was shown that the combination of two oncogenes such as *ras* and *myc* greatly enhanced *in vitro* transformation of normal embryonic fibroblasts (Land et al., 1983a, b), though *ras* oncogene alone was effective to induce complete or partial transformation in several types of primary cells (Holmes et al., 1986; Land et al., 1983b; Lichtman et al., 1986). Therfore, introduction of second oncogene such as *myc* or ElA, for example, into *ras*-transfected DC may result in the transformation of the DC. Another attempt to be tested is the transduction of oncogene(s) into DC precursor cells. Since DC are bone marrow-derived cells (Steinman et al., 1974), oncogene-transfected bone marrow cells, for example, may produce DC transformants during *in vivo* differentiation when they are added back into syngeneic mice. It seems also tempting to utilize another viral packaging system, ψ-AM (Cone & Mulligan, 1984), since it has wider range of target cell infectivity than ψ2 system and is expected to be a more efficient transformation system after introduction of oncogenic retroviral vectors, as suggested by Lichtman et al. (1986) from the transformation study of murine B lymphocytes using amphotropic helper MuLV. We are now planning these experiments.

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