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Abelson Virus Transformation of an Interleukin 2-Dependent Antigen-Specific T-Cell Line

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Abelson murine leukemia virus (A-MuLV) carries the gene v-*abl*, one of a group of oncogenes with structural and functional (tyrosine kinase) homology to three growth factor receptors. Work in this and other laboratories has shown that A-MuLV infection can render myeloid and lymphoid cells independent of the growth factors interleukin 3 and granulocyte-macrophage colony-stimulating factor. We have now shown that v-*abl* can also relieve interleukin 2 (IL-2) dependence in T cells. We infected a cloned IL-2-dependent antigen-specific cell line. Transformed cells were generated which were factor independent and tumorigenic. The transformants each bore unique v-*abl* DNA inserts and expressed v-*abl* mRNA. No elevation of expression of either IL-2 or its receptor could be detected in these cells. Thus, A-MuLV can short-circuit the dependence of hematopoietic cells on IL-2, IL-3, and possibly granulocyte-macrophage colony-stimulating factor, none of whose receptors are known to be of the tyrosine kinase type.

The cell line A3 was derived from the spleen of an A/J mouse which had been immunized with azobenzene arsonate-coupled syngeneic spleen cells as described previously (6). The clone A3.37.4 (A4) was derived after 6 months of culture in the presence of antigen and interleukin 2 (IL-2). It produced IL-2, interleukin 3 (IL-3), and gamma interferon (IFNγ) in response to azobenzene arsonate-coupled cells or the mitogen concanavalin A (ConA). The cell surface phenotype of the line was typical of class II restricted helper T cells, i.e., Thy-1$^+$, L3T4$^+$, Ly-2$^-$. Moreover, although the clone was strictly IL-2 dependent for growth, its expression of IL-2 receptor was constitutive and it no longer required repeated antigen stimulation.

Three infection protocols were used. No transformants were obtained by a conventional method of incubation with supernatants harvested from the Ann1 cell line which had been superinfected with Moloney murine leukemia virus (Mo-MuLV) and thus produced wild-type Abelson murine leukemia virus (A-MuLV) (1,3).

It has been reported by others that normal stimulated spleen T cells are refractory to infection by murine ecotropic retroviruses, such as Mo-MuLV. There is some evidence that this is a result of cell surface interference by endogenously coded viral envelope proteins (12, 14). Therefore, we modified the infection conditions to favor virus-cell fusion by removing serum from A(MoMuLV) preparations by ultracentrifugation through sucrose and from cell surface proteins by trypsinization, followed by the addition of polyethylene glycol 1000 prepared as described by Yoakum et al. (45). Suspensions of cells with virus were incubated at 37°C in 10% CO_2 for 2 h with occasional gentle swirling. Cells were diluted to a concentration of 10^5/ml in medium containing 15% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 2 mM fresh glutamine, and 5% ConA-stimulated spleen cell supernatant (10) as a source of IL-2. Suspensions were plated in 24-well tissue culture plates (1.5 ml per well) and reincubated. Medium lacking IL-2 was used to feed the cultures on day 2 and then at weekly intervals.

Factor-independent cell colonies, which were detectable at 2 to 4 weeks, were expanded and maintained in IL-2-free medium. Infection with A(MoMuLV) by this protocol generated IL-2-independent transformants at a frequency of approximately 10^{-6} (Table 1).

However, simple cocultivation with virus producer cell lines elevated this frequency markedly. Two virus-producing cell lines were used: the Ann1 line, producing A(MoMuLV), and Aψ, a clone of the ψ2 line (20) which carries a replication-defective Mo-MuLV vector genome and had been transfected with the p120 A-MuLV genome (35). Equal numbers (1×10^6 to 5×10^6) of target cells and irradiated (3,000 rads) producer cells were cocultivated for 2 h at 5×10^6 cells per ml in medium containing polybrene (4 μg/ml). After dilution to 5×10^5/ml and culture for a further 48 h in medium containing an optimal concentration of IL-2, cells were washed, plated at limit dilution in 0.2-ml culture wells, and maintained as for supernatant infections. When Mo-MuLV-superinfected Ann1 cells were used as producers, this method yielded a transformation frequency of approximately 10^{-3} (Table 1). Furthermore, cocultivation with Aψ allowed highly efficient infection; 30% of cells plated generated IL-2-independent clones (Table 1). A second cultivated helper T-cell line, P41.1, was transformed at a frequency of 10% by the Aψ cocultivation protocol (data not shown). The 100-fold-higher efficiency of the Aψ virus could be explained by the combination of the lack of competition for cell surface receptors by helper virus and the higher titer of A-MuLV produced by the Aψ cells.

Furthermore, the frequency of Aψ virus-generated IL-2 independence was comparable with the frequency of neomycin-resistant transformants generated by Uchida et al. (37) with the same ψ2 retrovirus packaging cell line and helper-type T-cell lines similar to A4 as targets. This argues strongly that in our experiments most or all A-MuLV-infected cells were rendered IL-2 independent.

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TABLE 1. A-MuLV induction of factor-independent cell lines[a]

Virus	Titer	No. of cells infected	Frequency of IL-2-independent clones
Supernatant infections			
A(MoMuLV)	2×10^6	2.4×10^7	9×10^{-7}
None		2.4×10^7	$<4 \times 10^{-8}$
Mo-MuLV	5×10^6	4×10^7	$<2.5 \times 10^{-8}$
Cocultivation			
A(MoMuLV)	2×10^4	2×10^7	1×10^{-3}
A(ψ-MoMuLV)	2.4×10^5	2×10^6	3×10^{-1}
Mo-MuLV	5×10^5	1×10^6	$<1 \times 10^{-6}$

[a] Virus titer is expressed as BALB/c 3T3 focus-forming units for A-MuLV (with or without helper virus) and XC plaque-forming units for MoMuLV. A(MoMuLV) was produced by the pI20 Ann1 cell line which had been superinfected with Mo-MuLV (3). A(ψ-MoMuLV) was produced by Aψ, a ψ2 line (20) transfected with the pI20 A-MuLV genome (35). Mo-MuLV was produced by an infected 3T3 line (3).

A total of 2.4×10^7 uninfected A4 cells and 4×10^7 cells exposed to Mo-MuLV failed to yield any factor-independent growth (Table 1). Moreover, no spontaneously arising IL-2-independent or tumorigenic cells have been detected in multiple in vitro and in vivo tests over a period of 2 years. This suggested that A-MuLV infection was essential in the generation of IL-2 independence in this cell line.

For further evidence of the involvement of A-MuLV in this process, we established that the viral DNA was integrated and expressed in all the IL-2-independent clones. DNA was extracted, cleaved by *Hin*dIII, electrophoresed in agarose in Tris-borate-EDTA buffer, and transferred to a nitrocellulose filter by standard procedures (19). After hybridization at 41°C in 50% formamide and 3 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), filters were washed at 55°C in 0.1× SSC and exposed to X-ray film. This analysis of DNA from seven separate factor-independent populations revealed one to two unique *abl* insertions in addition to the c-*abl* sequence in each cell line (Fig. 1A). The v-*abl* insert was expressed, as shown by Northern gel analysis of RNA from three of these lines (Fig. 1B). Polyadenylated [poly(A)$^+$] RNA was prepared and analyzed by formaldehyde-agarose gel electrophoresis as described by Gonda et al. (9). In all cases 4 μg of poly(A)$^+$ RNA was loaded per track. Whereas the factor-dependent A4 transcribed only low levels of the c-*abl* mRNAs (40) (not visible at this exposure level), each transformed line showed the 5.1-kilobase (kb) v-*abl* mRNA (formerly though to be 5.8 kb [32]) in abundance.

Full-length and subgenomic *env* mRNAs of the Mo-MuLV helper virus (32) were revealed in only one of three A4 A(MoMuLV) transformants examined here (Fig. 1C). None of eight other A(MoMuLV) transformants tested expressed infectious virus detectable by standard XC cell assays (data not shown). This is in contrast to the expression patterns of A(MoMuLV)-transformed thymocytes (from thymomas induced by intrathymic inoculation [3]) and FDC-P1 myeloid cells (transformed from IL-3 or granulocyte-macrophage colony-stimulating factor [GM-CSF] dependence in vitro) (4). In most examples of these studied, Mo-MuLV was shown to be expressed. The difference may simply reflect poor replication of Mo-MuLV in A4 cells.

Five of the transformants were tested for their tumorigenic potential by subcutaneous injection of 10^7 cells into syngeneic mice. All five transformed lines produced tumors at the site of injection with a latency of 14 to 30 days, with later spread to lymph nodes, spleen, liver, and kidneys (data not shown). In contrast, eight mice injected with 10^7 uninfected A4 cells showed no sign of tumors over a 6-month observation period.

To confirm the identity of the transformed cells, i.e., to exclude the possibility of cross-contamination of cultures, we performed Southern blot analysis of their T-cell receptor (TCR) β-chain genes (11). The transformants, including one line recovered from a subcutaneous tumor, each showed the diagnostic pattern of β_1 gene rearrangement displayed by the uninfected clone (Fig. 2A). Moreover, the transformants continued to express both the TCR α-chain (43) (Fig. 2B) and the β-chain (11) (Fig. 2C) mRNAs, although at reduced levels. The transformants also continued to express the normal helper cell surface phenotype (Thy-1$^+$ L3T4$^+$ Lyt-2$^-$) and responded to mitogen or specific antigen with a burst of production of lymphokines, including IL-2.

Since the A-MuLV transformants were IL-2 independent, we were obliged to ask whether constitutive endogenous production of this factor played any role in transformation.

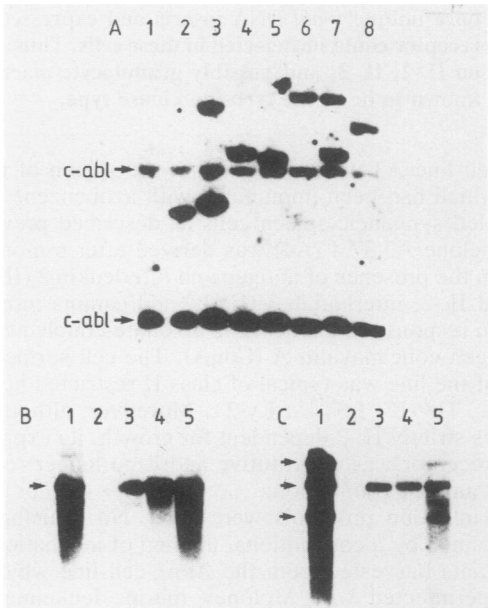

FIG. 1. A-MuLV is contained and expressed by the transformed cells. (A) Southern gel analysis of DNA. *Hin*dIII-digested DNA was electrophoresed in a 0.8% agarose gel and blotted onto a nitrocellulose filter which was probed with a v-*abl*-specific probe. Tracks: 1, A4 (uninfected); 2 to 8, A-MuLV-transformed A4 lines (2, A4 *abl*.1 [tumor-derived]; 3, A4 *abl*.4; 4, A4 *abl*.10; 5, A4 *abl*.15; 6, A4 *abl*-II-1.1; 7, A4 *abl*-II-2.1; 8, A4 *abl*-II-2.3). The 8- and 3.4-kb fragments generated by *Hin*dIII cleavage of the c-*abl* gene (41) are indicated. (B) Expression of v-*abl* mRNA. Poly(A)$^+$ RNA was electrophoresed on a 1% agarose gel. The nitrocellulose blot was probed with a v-*abl*-specific probe. The predicted position of the v-*abl* mRNA (32) is indicated by the arrow. Poly(A)$^+$ RNA was from A-MuLV-induced thymoma cell line MK1 (track 1), A4 (uninfected) (track 2), and A-MuLV-transformed lines A4 *abl*.1 (track 3), A4 *abl*.15 (track 4), and A4 *abl*.4 (track 5). (C) Most A-MuLV-transformed A4 lines do not express Mo-MuLV. A poly(A)$^+$ RNA gel was probed with an Mo-MuLV long terminal repeat probe. The predicted positions of Mo-MuLV, v-*abl*, and Mo-MuLV *env* mRNAs are indicated (arrows, top to bottom). Tracks, Same as for panel B. The probe in A and B was a ^{32}P-labeled nick-translated 2-kb *Sma*I-*Hin*dIII fragment from the pAb3sub3 plasmid (8). The probe for C was a 380-bp *Bam*HI-*Xba*I fragment cut from the pAm2 plasmid (35).

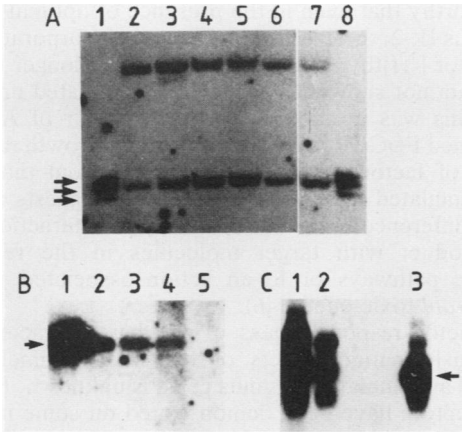

FIG. 2. T-cell receptor gene configuration and expression confirms the identity and mature phenotype of A-MuLV-transformed A4 cells. (A) *Pvu*II-digested DNA was electrophoresed in 0.8% agarose, and the nitrocellulose blot was hybridized with a probe containing the J and C regions of a murine TCR β cDNA. The 6.3-, 6.1-, and 5.9-kb germ line bands are indicated at the left (top to bottom). Tracks: 1, C57BL/6 liver; 2, uninfected A4; 3 to 7, A-MuLV-transformed A4 lines (3, A4 *abl*.4; 4, A4 *abl*.10; 5, A4 *abl*.15; 6, A4 *abl*-II-2.1; ;7, A4 *abl*.1 [tumor derived]; 8, C57BL/6 liver). (B and C) TCR α and β mRNAs are still expressed, although at much reduced levels, after transformation. Poly(A)$^+$ mRNA blots were hybridized with the TCR α (B) or β (C) probe. (B) Tracks: 1, uninfected A4; 2, A4 *abl*.1; 3, A4 *abl*.15; 4, A4 *abl*.4; 5, NIH 3T3 fibroblasts. The position of the 1.5-kb α mRNA (43) is indicated. (C) Tracks: 1, uninfected A4; 2, A4 *abl*.1; 3, VL_3, a radiation leukemia virus-induced thymoma cell line. The 1.3-kb correctly rearranged β transcript is indicated. The TCR β probe was a 0.6-kb *Eco*RI fragment cut from the 86T1 plasmid (11). The TCR α probe was a 1.1-kb *Eco*RI fragment cut from the py14 plasmid (43).

Supernatants of both uninfected and transformed A4 cells cultured at 10^6/ml showed less than 0.04 U of IL-2 activity per ml (7), compared with 320 U/ml from a standard preparation of ConA-stimulated spleen cells. Northern gel analysis (Fig. 3) showed that although a low level of constitutive IL-2 mRNA was detectable in the parent clone, there was no elevation of production of IL-2 mRNA, either constitutive (tracks 1, 2, and 3) or mitogen-induced (tracks 4, 5, and 6) in the transformants, but instead a decrease, so that no IL-2 mRNA was detectable in the uninduced cells. This effect was not specific to IL-2 mRNA, since both TCR α and β mRNA levels were also depressed (Fig. 2B and C), whereas β2-microglobulin mRNA levels were unaltered (data not shown).

The number per cell and affinity of IL-2 receptors were also assayed. Purified recombinant human IL-2 (kindly provided by Biogen SA, Geneva, Switzerland) was radioiodinated by the procedure of Robb et al. (28) with slight modifications. Briefly, 10 μg of IL-2 was mixed with 1 mCi of $Na^{125}I$ and 90 μg of chloramine T for 30 s at room temperature in a total volume of 100 μl of phosphate-buffered saline. The reaction was stopped with 100 μl of sodium metabisulfite (2.4 mg/ml). [^{125}I]IL-2 was separated from free $Na^{125}I$ on a Sephadex G25 column. Recovered [^{125}I]IL-2 was 95% precipitable by trichloroacetic acid and had a specific activity of 1.5×10^6 cpm/pmol. Approximately 20% of the [^{125}I]IL-2 could be bound in cellular excess.

The IL-2 equilibrium binding assay of Robb et al. (29) was used with minor modifications (17) on cells harvested from exponentially growing cultures of uninfected and transformed A4 cells. Nonspecific binding was determined by using an excess (21 μg/ml) of an anti-IL-2 receptor monoclonal antibody, PC61, which completely inhibits IL-2 binding at 4°C (18). Scatchard data were analyzed with a weighted least-squares curve-fitting program (22). A two-site binding model was chosen by the computer as the best fit for the experimental data.

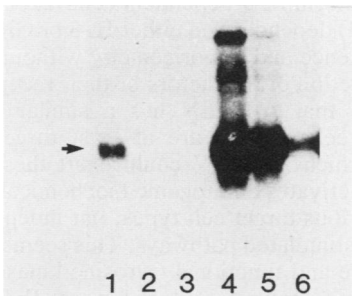

FIG. 3. A-MuLV transformation does not induce expression of IL-2. Poly(A)$^+$ RNA was electrophoresed in 1% agarose and hybridized with a murine IL-2 probe. Tracks: 1 and 4, A4 (uninfected); 2 and 5, A4 *abl*.1; 3 and 6, A4 *abl*.1 (tumor derived); 1 to 3, untreated; 4 to 6 conA stimulated. The position of the 1-kb IL-2 mRNA is indicated. The probe was a 0.5-kb *Pst*I fragment cut from the pMIL-2-20 plasmid (15). Cells were stimulated for 6 h with ConA (5 μg/ml) as described previously (6).

This analysis of IL-2 receptors showed no substantial change in the affinity of either high- or low-affinity sites (Fig. 4). The number of binding sites per cell was also unaffected: the transformant A4abl.1 bore 4.1×10^5 low-affinity and 3.7×10^3 high-affinity sites, compared with the factor-dependent line A4, which displayed 5.3×10^5 and 6.9×10^3 of the respective receptors. The differences in the number of each type of receptor for the two cell lines were not significant given the error of the method (29).

Consistent with their retention of IL-2 receptors, the transformants did retain some responsiveness to IL-2 (Fig. 5). This has been studied in detail and will be reported elsewhere (manuscript in preparation). However, the cells continued to proliferate in the absence of the exogenous factor (Fig. 5).

It has been demonstrated in this laboratory and others that

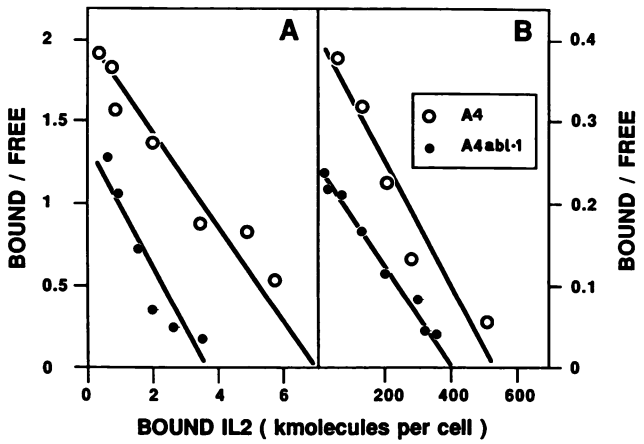

FIG. 4. Scatchard analysis of equilibrium IL-2 binding to transformed (A4 *abl*.1) and control (A4) T-cell lines. Panels A and B represent analysis of high- and low-affinity binding, respectively.

A-MuLV can transform IL-3-dependent cells (21, 24, 26) and a cell line (FDC-P1) dependent on either IL-3 or GM-CSF (4) to factor independence and tumorigenicity without inducing or elevating production of the factors or their receptors. We have shown here that A-MuLV has a similar effect on Il-2-dependent T cells. There are at least three possible mechanisms by which A-MuLV could exert these effects. One is that v-*abl* activates a mitogenic biochemical pathway common to its various target cell types, but independent of the growth factor-stimulated pathways. This seems unlikely, given the sequence and functional (tyrosine kinase) homology between *abl* and known receptors for growth factors (5, 13, 31, 38, 44), although not those for IL-2 (16, 23, 33), GM-CSF (39), or IL-3 (25; N. Nicola, personal communication). The second is that v-*abl* has targets within each of at least two separate mitogenic pathways. It is known, for example, to phosphorylate tyrosines on several intracellular proteins (30, 34, 42). The third is that the intracellular biochemical pathways activated by at least the IL-2 and IL-3 receptors have one or more reactants in common, including a target for the action of the v-*abl* product. In this respect, it is noteworthy that even in the presence of optimal doses of exogenous IL-2, the T-cell transformants incorporated lower amounts of [^{3}H]thymidine (Fig. 5) and had longer doubling times (data not shown) than the IL-2-stimulated uninfected clone. This was in contrast to the behavior of A-MuLV-transformed FDC-P1 myeloid cells, whose growth rate in the absence of factor was indistinguishable from that of the factor-stimulated uninfected cells (4). It suggests a tissue-specific difference, either in efficiency of interaction of the v-*abl* product with target molecules in the respective mitogenic pathways or in an action associated with the known v-*abl* toxic effect (46).

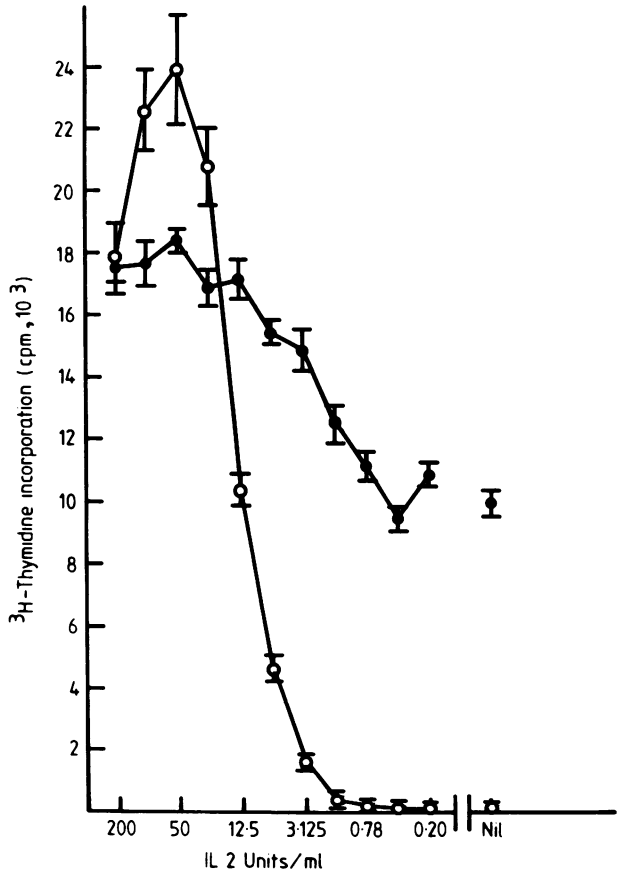

FIG. 5. A-MuLV-transformed A4 cells are independent of but responsive to exogenous IL-2. A4 (○) and A4 *abl*.1 (●) were cultured in the present of recombinant IL-2, and [^{3}H]thymidine incorporation was determined on day 3. Cells were cultured at 10×10^4/ml in 100 μl of RPMI 1640 medium supplemented with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, and recombinant human IL-2 (Cetus Corp.) at the concentrations indicated. After 72 h, cultures were pulsed for 6 h with 1 μCi of [^{3}H]thymidine (specific activity, 20 to 30 Ci/mmol; Amersham), and the radioactivity incorporated was determined by scintillation spectrometry. Vertical bars indicate the standard error.

The factor responsiveness of the best-characterized in vivo transformation targets of this virus, namely pre-B lymphoid and immature thymus cells, is unknown. However IL-2 receptors have been demonstrated on some immature thymocytes (2, 27) and activated B cells (36, 47). Also, myeloid cells, which can be transformed in vivo by this virus, were stimulated by IL-3. Regardless of whether IL-2 and IL-3 are most relevant in vivo, the in vitro transformations described here and elsewhere strongly imply that at least part of the tumorigenic activity of A-MuLV in vivo is due to a short circuit of the biochemical pathway(s) which normally mediates mitogenic responses to more than one growth factor.

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