Abrogation of the Requirement for Feeder Cell Interaction and T Cell Receptor Stimulation of Lymphocytes Infected with Retroviral Vectors

By Christine Laker,* Gisela Gräning,* Anne Kelso,‡ Carol Stocking,* and Wolfram Ostertag*

*From the *Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, 2000 Hamburg 20, Federal Republic of Germany; and ‡The Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, Victoria 3050, Australia*

Summary

Infection of sensitive adult mice with myeloproliferative sarcoma virus (MPSV) results in a myeloproliferative syndrome. Two components of the viral genome are required to induce this unique pathology: the *mos* oncogene and sequences within the U3 region of the long terminal repeat (LTR). In studies designed to identify the target cell of MPSV and thus better understand the mechanism by which a myeloproliferative syndrome is induced, we have infected a series of T cell lines with MPSV-based vectors. The results presented here show that infection with neo^R MPSV abrogates the requirement for an antigen-specific or feeder cell–dependent stimulation, without altering the requirement for interleukin 2. Significantly, this response is not dependent on the *mos* oncogene, but requires sequences within the U3 region of the MPSV LTR. No alteration in the constitutive or induced levels of lymphokines released by these cells was observed. These results suggest a model in which T cells acquire a proliferative advantage by uncoupling the proliferative response from the lymphokine synthesis that is induced by activation of the T cell receptor. These cells are thus poised for antigen stimulation and secretion of cytokines that stimulate myelopoiesis.

The myeloproliferative sarcoma virus (MPSV)[1], a member of the Moloney murine sarcoma virus (Mo-MuSV) family, differs from all other members of this *mos*-oncogenic virus family by its distinct ability to induce a myeloproliferative disease in adult $Mpsv^s$ mice (1, 2). Recombinant viruses between MPSV and Mo-MuSV have demonstrated that the pathology induced by MPSV requires both the *mos* oncogene and unique sequences within the MPSV U3 region of the long terminal repeat (LTR) (3, 4). In addition, results obtained from comparing the efficiency of expression of MPSV and Moloney murine leukemia virus (MuLV)-derived vectors in hemopoietic precursor (5) and stem cell lines (Beck-Engeser, G. et al., manuscript submitted for publication), lymphoid precursor cells (6), liver cells (7), and the embryonic carcinoma cell lines F9 (8) and PCC4 (9) have established that the MPSV LTR is a more effective transcriptional promoter than the Mo-MuLV LTR in these cells. The myeloproliferative disease induced by MPSV is thus most likely a consequence of relatively high levels of *mos* expression in a specific target cell(s). The target cell for the MPSV-induced myeloproliferative disease has, however, remained elusive.

MPSV infection of adult $Mpsv^s$ mice generates a large increase (in some cases >100-fold) in hematopoietic precursor cells, including all cells of the myeloid lineage (eosinophils, megakaryocytes, erythroid, and macrophage-granulocyte precursors) and a large increase in myeloid stem cells as measured by spleen colony formation (CFU-S), and by mixed colony assays (10, 11). Two alternative explanations for the large increase in precursor cells in MPSV-infected animals have been proposed (1, 12). The simplest interpretation would be that all of the infected precursor cells have gained a proliferative advantage as a consequence of increased expression of the *mos* oncogene. However, when established hematopoietic multipotent and precursor cell lines (e.g., FDC-Pmix and FDC-P1) were infected with MPSV, and their proliferation and relative growth factor response monitored, no significant differences in growth kinetics or factor requirements were observed, as compared with uninfected controls (5, and un-

[1] *Abbreviations used in this paper:* CM, conditioned medium; GM-CSF, granulocyte/macrophage-CSF; GTU, G-418 resistance transfer units; LTR, long terminal repeat; MOI, multiplicity of infection; Mo-MuSV, Moloney murine sarcoma virus; MPSU, myeloproliferative sarcoma virus; MuLV, Moloney murine leukemia virus; SN, supernatant.

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published results). A more sophisticated model to account for the viral-induced myeloproliferation has been proposed based on the observation that spleens from infected mice release a growth factor or CSF that stimulates myeloid precursor cells, and thus could account for the myeloproliferation (13). Support for this alternative has also been provided by experiments using a mutant of the MPSV, in which the transforming function of the *mos* oncogene is temperature sensitive (14). Spleen cell–conditioned media (CM) from MPSV-infected mice sustained erythro- and myelopoiesis in vitro only if prepared under permissive conditions for the *mos* oncogene (15). The disease could thus be due to increased CSF release, as has been described in mice injected with multipotent hematopoietic cells expressing IL-3 or granulocyte/macrophage (GM)-CSF via a retroviral vector (16, 17).

As lymphocytes are one of the major sources of growth factors, they are a potential target for the action of MPSV. $CD4^+$ lymphocytes release a broad variety of lymphokines acting on the immune response as well as on myelopoiesis (e.g., IL-3, GM-CSF, IL-4, IL-5, IL-6). However, production of lymphokines requires either prior activation via the TCR by antigen presentation or by antibodies that recognize the Ti or CD3 components of the TCR complex (18). Lectins like Con A can activate T cells by binding polyvalently to the TCR, and thus are functionally equivalent to antigen and anti-TCR antibodies (19).

In this work, we wanted to analyze the effect of MPSV infection on lymphocytes with regard to growth requirements and cytokine production. Three murine T cell lines with different requirements for continued growth were used as model populations: S49IG3 cells (20), LB3 (21), and E9.D4 (22). The E9.D4 cell line requires both IL-2 and TCR activation by a specific hapten-Ia complex for a mitogenic response. The LB3 cell line also has a strict requirement for two stimuli for proliferation: IL-2, and a stimulus presented to the cell via spleen filler cells. The nature of the spleen filler cells required for the maintenance of both T cell lines E9.D4 and LB3 is unknown. Although the LB3 cell line was derived from a primary BALB/c anti-DBA/2 MLC, LB3 cells have been shown to be of DBA/2 origin (own unpublished results), and thus, the mechanism of activation by the syngeneic spleen cells is unknown. The third cell line, S49IG3 lymphoma, is neither IL-2 dependent nor requires filler cells for proliferation. All lines can be induced to express several cytokines that stimulate myelopoiesis (23, 24).

In this study, we focused on the putative transforming potential of MPSV on T cells after infection by investigating: (*a*) IL-2 requirement for growth; (*b*) requirement for stimulation by antigen or by an undefined soluble factor; and (*c*) lymphokine production. Three types of vectors were used to test separately the role of the *mos* oncogene and of the unique transcriptional control region of MPSV.

Materials and Methods

Cell Lines. All T cell lines and the myeloma cell line NSO were maintained in modified DMEM, L-glutamine, and 5–10% heat-inactivated FCS (T cell medium). Supernatant (SN) (2–5%) of the phorbol ester (PMA)-induced EL4 thymoma cell line was used as a source of IL-2 (25). LB3 and E9.D4 cells were cultured by weekly passage with 3,000-rad irradiated spleen cells (DBA/2 and azobenzenearsonate-coupled CBA cells, respectively) as described (21, 22). Virus-producing cell lines were kept in modified MEM, L-glutamine (216 mg/liter), and 10% heat-inactivated FCS (27). All incubations were carried out at 37°C in a humidified atmosphere of 5% CO_2 in air.

Viral Infections. Infectious virus was collected as a 12-h SN from virus-producing Ψ2 cells (27) and filtered through prewashed 0.8-μm filters (Millipore Continental Water Systems, Bedford, MA). To assay for infectious virus particles, triplicate cultures of 10^3 NIH/3T3 fibroblasts/well were exposed to 1:5 serial dilutions of virus-containing SN. SN was removed after 24 h, and virus-infected cells were selected with medium containing 400 μg/ml Geneticin (G-418) and scored after 2 wk. The number of G-418-resistant colonies obtained at end-point dilution was used to calculate the G-418 resistance transfer units (GTU)/ml.

Lymphoid cell lines were infected by exposure to cell-free SN from virus-producing Ψ2 cells supplemented with IL-2. After a 6-h incubation period, cells were washed two times, cultured in

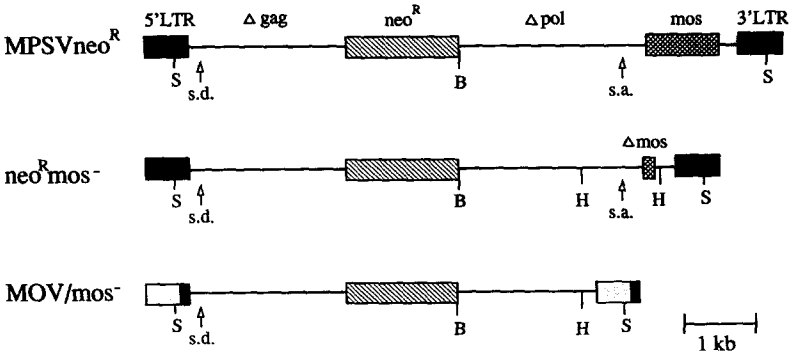

Figure 1. Vectors used for infections. Construction of neo^R MPSV and neo^R/mos$^-$ have been previously described (3). Note that the residual *mos* sequences of neo^R/mos$^-$ potentially encode a 59-residue peptide sharing only 29 NH_2-terminal amino acids with the 300-residue *mos* protein, but no transforming activity in either in vitro or in vivo assays has been demonstrated. MOV/mos$^-$ was constructed by replacing a ClaI × BglII fragment containing the 3'MPSV LTR from neo^R/mos$^-$ with a similar fragment containing the 3'Mo-MuLV LTR (cl MOV3; 47, and M. Grez, unpublished results). A 1.2-kb HindIII fragment containing residual *mos* and *pol* sequences was also deleted. After one round of replication, this vector is composed of chimeric LTRs, with the U3 region being derived from Mo-MuLV (indicated by a shaded box). Restriction enzymes used for vector construction and Southern analysis are abbreviated as follows: S, SstI; B, BamHI; and H, HindIII. s.d., splice donor; s.a., splice acceptor for the *mos* transcript.

T cell medium overnight, and plated the next day by limiting dilution in 96-well plates either with or without 1 mg/ml G-418. Cell concentrations plated ranged from 0.2 to 5 cells/well for uninfected control cells and 50 to 1,000 cells/well for infected cells. 2 wk after plating, positive wells were scored. Cloning efficiency was calculated by Poisson distribution analysis.

Replica Plating Assay. Half the media and cells (100 μl) of positive wells from primary infection plates was transferred into 96-well plates and supplemented with T cell medium containing 20% FCS, 4–10% EL4 SN, and where indicated, 2 mg/ml G-418. Filler cells were not added as a stimulus. Transfer of positive wells was carried out once a week.

IL-2-dependent Proliferation Assay. Cells were washed three times before a 48-h incubation with 1:3.15 serial dilutions of EL4 SN as a source of IL-2 in 96-well plates (10^4 cells/well). After a 6-h pulse with 0.5 μCi [^{3}H]thymidine/well, triplicate cultures were harvested onto glass fiber filters with an automatic cell harvester (Skatron, Norway). Incorporated radioactivity was measured by scintillation spectrometry. The IL-2-dependent T cell line CTLL was used as standard control.

CSF Production Assay. Cells were washed three times and cultured at 5×10^5 cells/ml with or without anti-CD3 antibody (145-2C11) adsorbed to culture wells as a 10-μg/ml solution in PBS as previously described (24). After 48 h of incubation, CM were harvested and assayed for GM-CSF activity on FDC-P1 and FDC-P2 or 32D cells (2×10^3 cells/well) by [^{3}H]thymidine incorporation. Mouse lung cell CM (2.5×10^4 U/ml) or rIL-3 (1 ng/ml = 320 U/ml) were used as internal standards.

RNA and DNA Analysis. Genomic DNA was isolated by standard techniques, and restriction enzyme digestions were performed as recommended by the suppliers. Digested DNA was separated on agarose gels and transferred to GeneScreen Plus (New England Nuclear, Boston, MA) membrane filters. Total RNA was isolated by the procedure of Auffray and Rougeon (28), denatured with glyoxal and dimethylsulfoxide, and subjected to electrophoresis through an agarose gel (29) before transfer to membrane filters. DNA or RNA immobilized on filters were hybridized with DNA fragments labeled by the method of Feinberg and Vogelstein (30). Probes used for analysis included *mos*- and *neo*-specific sequences previously described (3).

Results

Equal Transfer Efficiencies of MPSV- and Mo-MuLV-derived Vectors into Lymphoid Cell Lines. We first sought to determine if the different lymphoid cell lines could be effectively infected and if any difference in infection efficiency could be seen between several vectors. Three MPSV-based vectors containing the neomycin phosphotransferase gene (neo^R) as a dominant selectable marker were used for infection (Fig. 1). neo^{R}MPSV is the wild-type vector with the intact *mos* oncogene, whereas in neo^R/mos$^-$, the *mos* coding region has been deleted. In a third construct the LTR U3 region of the neo^R/mos$^-$ vector has been replaced with sequences of the Mo-MuLV (MOV/mos$^-$). For general comparison, the Mo-MuLV-derived Zipneo^R vector (31) was also included. The Zipneo^R and the MOV/mos$^-$ both have the Mo-MuLV U3 region, in contrast to the other two constructs, which have MPSV-derived LTRs.

Vectors were introduced into Ψ2 packaging cells (27) to produce virus particles that transfer only the replication-defective vectors into the target cells. The two IL-2-dependent CD4$^+$ T cell lines, LB3 and E9.D4, as well as the thymoma cell line S49IG3 and the myeloma cell line NSO, were used as target cells for infection with MPSV vectors.

As shown in Table 1, neo^R transfer (GTU) of MPSV into all of the lymphoid cells was two or three orders of magnitude lower than transfer into NIH/3T3 fibroblasts. The transfer efficiencies of the two MPSV vectors, as compared with the two vectors with Mo-MuLV LTR U3 regions (Zipneo^R and MOV/mos$^-$), showed no significant differences.

Southern blot analysis was performed on DNA isolated from infected cultures to verify the presence of an integrated provirus. Digestions with SstI and BamHI in combination with neo- and *mos*-specific probes confirmed the presence of an intact provirus (Figs. 1 and 2, and data not shown).

T Cell Lines Infected with MPSV Are Unaltered in Their Requirement for IL-2. A model in which the paracrine stimula-

Table 1. *Transfer Efficiency of MPSV-based Vectors into Fibroblasts and Lymphoid Cells*

Cell line	Viral vector	U3 Region of the LTR	*mos* Oncogene	Total GTU/Exp.		Ratio GTU (lymphoid cells/fibroblasts)
				Fibroblasts	Lymphoid cells	
LB3	neo^R MPSV	MPSV	+	1.4×10^5	5.5×10^3	3.9×10^{-2}
	neo^R MPSV mos$^-$	MPSV	–	1.8×10^4	4.5×10^2	2.5×10^{-2}
	neo^R MOV/mos$^-$	Mo-MuLV	–	3.4×10^3	1.8×10^2	5.3×10^{-2}
E9.D4	neo^R MPSV	MPSV	+	5.6×10^5	6.7×10^2	1.2×10^{-3}
	neo^R MPSV mos$^-$	MPSV	–	5.5×10^5	1.1×10^3	2×10^{-3}
	MZip neo^R	Mo-MuLV	–	6.3×10^3	7.5×10^1	1.2×10^{-2}
S49IG3	neo^R MPSV	MPSV	+	1.2×10^4	9.7×10^2	8×10^{-2}
	neo^R MPSV mos$^-$	MPSV	–	1.6×10^6	5.7×10^3	3.6×10^{-3}
NSO	neo^R MPSV	MPSV	+	4.8×10^4	7.7×10^2	1.6×10^{-2}
	neo^R MPSV mos$^-$	MPSV	–	1.2×10^4	5.1×10^2	4.3×10^{-2}

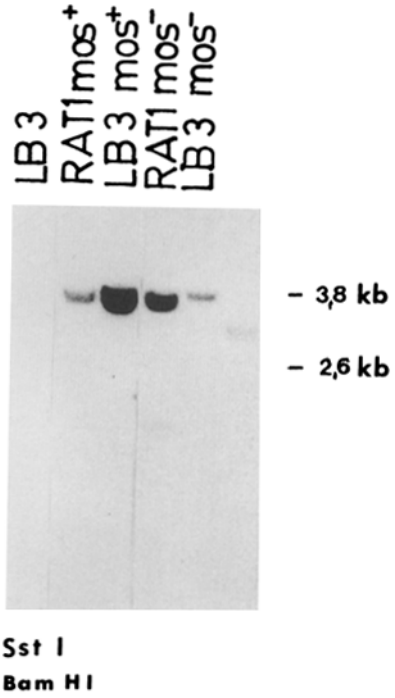

Figure 2. Southern analysis of infected LB3 cells. DNA of uninfected and infected LB3 cells and fibroblasts was digested with SstI and BamHI and hybridized to a neo^R-specific probe. Lane *1*, control, uninfected LB3; lane *2*, control, neo^R MPSV-infected fibroblast cell line; lane *3*, neo^R MPSV-infected LB3 cells; lane *4*, control, neo^R/mos$^-$-infected fibroblast cell line; lane *5*, neo^R/mos$^-$-infected LB3 cells.

Table 2. *Clonability of MPSV-infected LB3 Cells as a Function of Time after Stimulation*

Virus	Clone no.	Percent cloning efficiency at end-point dilution* 1‡	4	12
None	Bulk cultures	30–40	6	ND
neo^R MPSV	7§			59
	8			100
	9			59
	11			40
	12			39
				$\bar{X}$ = 60 ± 25
neo^R MPSV mos$^-$	1			69
	2			75
	3			39
	4			27
	5			30
				$\bar{X}$ = 48 ± 22

* Cloning efficiency was calculated by Poisson distribution.
‡ Weeks after stimulation.
§ Clones were obtained by recloning of virus-infected G418-resistant mass cultures.

tion of the myeloid compartment of MPSV-infected mice results in a myeloproliferative syndrome requires that there be a constant source of factor-producing cells. This could be provided by T lymphocytes that after infection with MPSV either have acquired altered mitogenic requirements and thus may be more readily induced to secrete growth factors or, alternatively, constitutively release high levels of growth factors without induction.

Therefore, we first tested whether LB3 or E9.D4 cells infected with neo^{R}MPSV acquired IL-2-independent growth. The IL-2 requirement of infected LB3 and E9.D4 cells was assayed by [^{3}H]thymidine incorporation by proliferating cells in the presence of various dilutions of IL-2. Infected LB3 and E9.D4 cells maintained identical requirements for IL-2 to uninfected cells (~50 U/ml) and showed no altered sensitivity to IL-2, regardless of whether mos$^+$ or mos$^-$ vectors were used for infection. Thus, neither the *mos* oncogene nor retroviral infection alter the IL-2 requirement of these established T cell lines.

MPSV Infection Abrogates the Requirement of T Cell Lines for Filler Cells and/or Antigen Stimulation of the TCR. Some T cell lines periodically require a mitogenic stimulus provided by appropriate filler cells to maintain long-term proliferation in vitro. This stimulus may be either triggered directly via the TCR, as in the case of the hapten-specific antigen-dependent E9.D4 cells, or alternatively, by an unknown mechanism, as in LB3 cells. Abrogation of this requirement by the *mos* oncogene may be a mechanism by which MPSV-infected lymphocytes could acquire a selective growth advantage in vivo. We therefore tested whether the altered growth properties of the IL-2- and feeder cell–dependent LB3 cells were altered after infection with neo^R MPSV. Under optimal conditions (weekly exposure to irradiated spleen cells), the cloning efficiency of the uninfected parental LB3 cell line varied between 30 and 40%, but decreased to 6% 4 wk after the last stimulation; soon afterwards, the clone (or the cells) became extinct (Table 2). Uninfected LB3 mass cultures could not be propagated for >12 wk without stimulation. In contrast, MPSV-infected LB3 cultures exhibited a cloning efficiency of 60% 10 wk after infection and 12 wk after the last stimulation with filler cells (Table 2). This cloning efficiency exceeds that of the uninfected parental LB3 cells maintained under optimal conditions.

Clonal analysis of different infected subclones provides a more definitive test to measure the relative growth advantage and feeder independence of infected lymphocytes. Thus, a second series of experiments was designed to analyze several hundred clones. The E9.D4 cell line was included in this

analysis to determine if MPSV infection would also lead to abrogation of the requirement for antigenic stimulation. Cells infected with a multiplicity of infection (MOI) of <1 were plated in microtiter plates under G-418 selection at cell densities that ensured the outgrowth of a single infected cell per well, based on Poisson distribution analysis. The resulting infected clones were grown to confluence and then diluted 1:2 for each serial passage in a second microtiter plate. Clonal extinction of ~500 clones each of uninfected and neo^R MPSV-infected T cells was monitored over a several-month period under identical growth conditions and comparable initial cell densities (Figs. 3 and 4). The replica plating assay was started 2 wk after infection and selection for virus-positive (G-418 resistant) clones.

As shown in Fig. 3, the number of surviving uninfected LB3 clones decreased dramatically during the first and second platings to ~20% of the original number of plated clones, finally decreasing to <5% during the time course of the experiment. Significantly, attempts to expand individual clones after the sixth and seventh plating in search of spontaneous stimulation-independent mutants failed. The number of infected G418-resistant clones dropped as well during the first two platings, although to a lesser extent (60 and >40%, respectively). Numbers of clones reached a plateau phase of 30–40% of the number of clones initially plated until more stress was applied after the fifth replating by transferring only 25% of the cells into new wells. Clone numbers were quickly reduced to 5–10% of clones seeded at the starting point of the experiment. Replica plating experiments with E9.D4 cells (Fig. 4) were similar to those described for LB3, except that no added stress was applied by replating fewer cells at any passage. After 12 passages, a final plateau of 17% surviving neo^R MPSV-infected E9.D4 was reached. These clones could be replated for almost 1 yr (46 transfers), until termination of the experiment.

As infected, but not uninfected, LB3 and E9.D4 cells were selected in the presence of G-418 in the replica plating assay, a control for a possible drug effect on cellular metabolism and doubling time was carried out. After the second passage, LB3 and E9.D4 clones selected in G-418 were passaged into medium without G-418, and the remaining cells were refed with selection medium. Virus-positive clones maintained without G-418 displayed a pronounced improvement of survival (20% in LB3 clones, 12% in E9.D4 clones) over the identical population maintained under selective pressure (Fig. 5). G-418 selection, thus, does not augment but rather counteracts the effect induced by MPSV infection.

The mos Oncogene of MPSV Is not Necessary for Filler Cell–dependent Growth of MPSV-infected T Cell Lines. Experiments described in the previous section show that MPSV infection abrogates the requirement for filler cells (LB3 cells) and also for antigen-TCR interaction (E9.D4 cells). Clonal analysis

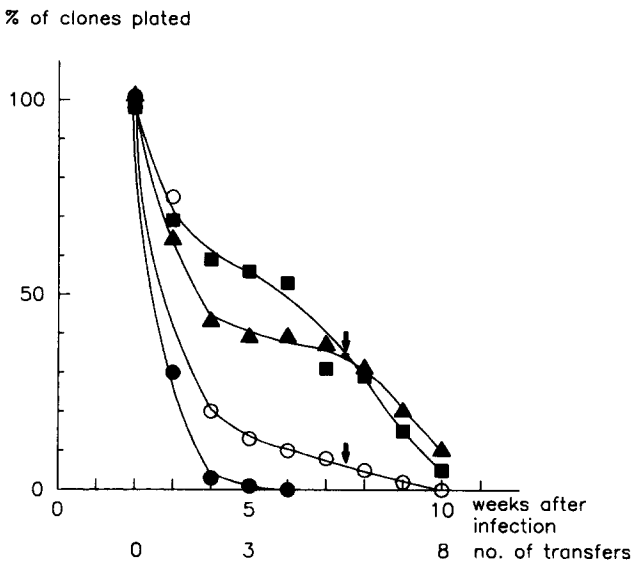

Figure 3. Filler cell–independent growth of MPSV-infected LB3 cells. Replica plating assay of uninfected and infected LB3 clones. Infected clones were obtained from primary infection plates at end-point dilution after 2 wk of G418 selection (1 mg/ml) for virus-positive clones. Uninfected LB3 clones were obtained from control experiments to determine cloning efficiency. Clones were maintained in the presence of EL4 SN with (infected) or without (uninfected clones) G418 and in the absence of filler cells. Plates were scored weekly for proliferating clones, and half of the volume (100 μl) of positive wells was transferred into new 96-well plates, where the total volume was reconstituted with medium with 20% heat-inactivated FCS, EL4 SN, and with or without 2 mg/ml G-418. Percent values of clones plated were calculated from number of clones surviving 2 wk after infection and selection. Note that last exposure to filler cells occurred 2 wk before infection. Uninfected LB3 clones (○, n = 550), neo^R MPSV-infected LB3 clones (▲, n = 541), neo^R MPSV *mos*-infected LB3 clones (■, n = 541), neo^R MOV/mos$^-$-infected LB3 clones (●, n = 222). Arrows denote onset of cultivation stress, when only 25% (50 μl) of the volume of positive wells was transferred.

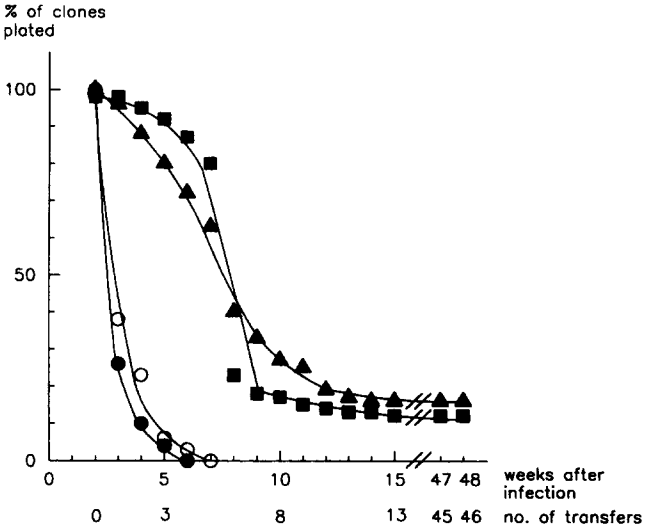

Figure 4. Filler cell–independent growth of MPSV-infected E9.D4 cells. Replica plating assay of uninfected and infected E9.D4 clones. Experiment was carried out as described in Fig. 3. Uninfected E9.D4 clones (○, n = 611); neo^R MPSV-infected E9.D4 clones (▲, n = 471); neo^R MPSV mos$^-$-infected E9.D4 clones (△, n = 592); mZip neo^R–infected E9.D4 clones (●, n = 87).

of mass cultures and replica plating of individual clones infected with the MPSV vector neo^R/mos$^-$ lacking the oncogene were carried out to test whether the *mos* oncogene was required for the abrogation of feeder dependence (Table 2 and Figs. 3 and 4). Results obtained from both mass culture and replica plating experiments of neo^R/mos$^-$-infected LB3 or E9.D4 cells showed a similar acquisition of filler cell–independent growth, as compared with cells infected with neo^R MPSV. The *mos* oncogene is thus not required for abrogation of the requirement for filler cells.

To ascertain that infection with MPSV affects clonability by interfering with the stimulation pathway, we infected the stimulation-independent thymoma cell line S49IG3 with either neo^R MPSV or neo^R/mos$^-$. End-point dilution experiments showed no enhancement in cloning efficiency after infection, regardless of the virus used for infection (15–22% both before and 5 wk after infection).

Filler Cell–independent Growth of MPSV-infected Cells Is a Property of the MPSV LTR and not a Result of Retroviral Infection. In previous work, we have pointed out that sequences within the U3 component of the MPSV LTR play a crucial role in the myeloproliferative disease (3, 4). To test the importance of the U3 region in the abrogation of the stimulation requirement, we infected LB3 and E9.D4 cells with vectors (Zipneo^R or MOV/mos$^-$) that contain the U3 region of the Mo-MuLV instead of MPSV LTR. Cells were infected, and >200 neo^R MOV/mos$^-$ LB3 clones and ~100 Zipneo^R-infected E9.D4 clones were selected and assayed by replica plating (Figs. 3 and 4). MOV/mos$^-$ and Zipneo^R clones were noticeably smaller at the time of plating, as compared with control neo^R/mos$^-$-infected clones, and total number of clones was dramatically reduced after the first transfer, resulting in <1% of the original number by the fourth and sixth transfer, respectively. Furthermore, no stable clones from either infection could be maintained in culture. No significant difference in proliferation of clones infected with Zipneo^R or MOV/mos$^-$, as compared with uninfected clones, was observed.

The marked difference in growth patterns of lymphocytes infected with virus containing either the MPSV or the Mo-MuLV LTR shows that the abrogation of filler cells and/or requirement for TCR stimulation is a property only of the vectors with an MPSV transcriptional control domain.

Lymphokine Production in T Cells Is not Altered after MPSV Infection. Do MPSV-infected lymphocytes release growth factors spontaneously or do they show alterations in growth factor induction that could account for a potential paracrine stimulation of myeloid cells in vivo? CM from uninfected and neo^R MPSV- or neo^R/mos$^-$-infected LB3, E9.D4, and S49IG3 cells were collected from either unstimulated or Con

Table 3. *Lymphokine Production of MPSV-infected T Cell Lines*

CM*		CSF activity	
Virus	Cell line	Unstimulated§	Stimulated‖
		U/ml	
None	LB3	29–250	5×10^3
	E9.D4	160	7.5×10^5
	S49IG3	0	ND
Neo^R MPSV	LB3 bulk culture	280	10^4
	clones (n = 6)	222 ± 125	ND
	E9.D4 bulk cultures		
	(n = 6)	39 ± 17	$5.9 \times 10^4 \pm 1.2 \times 10^4$
	S49IG3 bulk culture	0	ND
Neo^R MPSV mos$^-$	LB3 bulk culture	250	10^4
	clones (n = 9)	725 ± 715	ND
	E9.D4 bulk cultures		
	(n = 6)	20 ± 11	$9 \times 10^4 \pm 1.4 \times 10^4$
	S49IG3 bulk culture	0	ND

* CM was harvested after 48-h incubation of 5×10^5/ml washed cells, 5–10 wk after infection.
‡ CSF activity was measured on FDC-P1 cells by [^{3}H]thymidine incorporation. 50 U/ml is defined as the CSF activity that produces half-maximal stimulation of colony-forming cells in normal bone marrow. Internal standards of either mouse lung cell CM or rIL-3 was used for assays of LB3 and E9.D4 cells, respectively.
§ CSF activity of unstimulated cells vary ~10-fold from assay to assay depending on the timepoint of last exposure to filler cells (1–3 wk) before the experiment.
‖ Stimulation with either Con A (10 ng/ml) for LB3 or anti-TCR mAb 145-2C11 (5 μg/well, plastic-adsorbed) for E9.D4 cells.

A/anti-TCR–stimulated cells and tested for lymphokine production (Table 3). CSF activity in SNs from unstimulated, uninfected LB3 cells varied up to 10-fold, depending at which time point after last exposure to spleen filler cells (1–3 wk) the experiment was done. SNs from unstimulated MPSV-infected LB3 cells prepared 5–10 wk after infection showed a basal CSF activity within the same order of magnitude. CSF activity in CM from uninfected E9.D4 cells 1 wk after stimulation exceeded by four- to eightfold the activity detected in unstimulated, infected E9.D4 cells. Apparent differences were therefore not considered significant.

In addition, Con A or anti-TCR antibody stimulation of the infected T cell lines was not altered compared with the uninfected control. Uninfected controls, as well as infected LB3 and E9.D4 cells, showed a similar 50–5,000-fold increase in factor production of GM-CSF (Table 3) and similar levels of expression of IL-3 (data not shown), as previously described for uninfected LB3 and E9.D4 cells (23, 24).

Discussion

Release of growth factors that stimulate the hematopoietic cells of the myeloid compartment has been observed as a consequence of MPSV infection of adult mice and may be responsible for the myeloproliferative disease induced by MPSV (13, 14). Although several studies have concurred that properties of the LTR U3 region of MPSV, in addition to the *mos* oncogene, are required to induce the MPSV pathology (3, 14, 15), the target cell of MPSV has not been identified. The present work was designed to test if MPSV infection of lymphocytes would result in detectable alterations that would implicate these cells as the target cell of the MPSV pathology. Specifically, we asked: (*a*) whether growth properties and requirements of MPSV-infected lymphocytes are altered; (*b*) whether infected lymphocytes release growth factors constitutively or demonstrate an altered induction pattern compared with uninfected or control virus–infected lymphocytes; and (*c*) whether the U3 region of the MPSV LTR or the *mos* oncogene is required for any biological response observed. For this purpose, we infected three T cell lines that have different requirements for continued proliferation: one for IL-2, another one for IL-2 and an undefined interaction with splenic feeder cells, and a third with requirements for IL-2 and TCR stimulation.

The requirement for the lymphocytic growth factor IL-2 was unaltered in all of the three infected T cell lines regardless of whether the *mos* oncogene was present in the viral vector. Moreover, the dose response to IL-2 was unaltered. This finding is in contrast to many reports of other oncogenes that abrogate exogenous growth factor requirement or alter relative growth factor requirements in factor-dependent myeloid (32–35) or lymphoid cell lines (36, 37). Our results here are, however, consistent with our previous observations that the *mos* oncogene does not alter growth factor requirement of either myeloid precursor or stem cell lines (5, and unpublished results).

The observation that neither lymphocytes nor myeloid cells infected with MPSV exhibited altered growth factor requirements would suggest that MPSV does not directly abrogate factor dependence. Although some colony-forming cells of MPSV-infected mice do not require growth factors to form colonies, the number of such cells was disproportionate to the total number of cells plated in vitro, indicating that paracrine or autocrine stimulation, and not direct abrogation of growth factor requirements of infected cells, are involved in generating the myeloproliferative disease (38).

Significantly, however, the work presented here unequivocally shows that T cells infected with MPSV vectors no longer require antigen stimulation of the TCR, or in the case of the LB3 cell line, interaction with splenic feeder cells for long-term proliferation. Replica plating experiments allowed analysis on a clonal level and thus ruled out a possible outgrowth of spontaneous secondary mutants from mass cultures. Approximately 2,000 of either MPSV vector–infected or uninfected control clones were analyzed. No immortalization of uninfected clones was observed, whereas 20% of infected LB3 clones could be established as stable filler cell–independent clones. Approximately 15% of infected E9.D4 clones established in the replica plating assay survived almost 1 yr of weekly transfers (46 passages) until experiments were terminated.

One effect of TCR activation is the upregulation of both IL-2 and its receptor (39). It was therefore surprising that the requirement for IL-2 was unchanged after MPSV infection. Thus, MPSV-infected lymphocytes must express IL-2Rs at sufficient levels to ensure prolonged proliferation when stimulated with IL-2. Interestingly, we also did not detect any changes in the constitutive or induced levels of growth factor release in any of the T cell lines infected with either the MPSV or Mo-MuLV vectors compared with uninfected controls. Thus, the two TCR-dependent responses, mitogenesis and growth factor induction, have been uncoupled.

Quite unexpectedly, the *mos* oncogene was not necessary for stimulation-independent growth of infected cells, although it has been proven to be essential for the effect of MPSV in vivo. The continued growth without weekly stimulation of both LB3 and E9.D4 cells infected with the neo^R/mos^- control vector was equally efficient as in cells infected with neo^R MPSV.

However, consistent with previous results on other cells and on in vivo generation of myeloproliferative disease, sequences within the U3 region of the MPSV LTR are required for the altered growth requirements of infected lymphocytes. Exchange of the U3 region by Mo-MuLV sequences does not permit long-term propagation of the $CD4^+$ T cell lines without filler cell stimulation. Neither MOV/mos^--LB3–no $Zipneo^R$-infected E9.D4 cells survived the replica plating assay, disappearing with similar kinetics to the uninfected control cells.

By what mechanism do vectors containing the MPSV U3 LTR region, but not necessarily the *mos* oncogene, abrogate the requirement for filler cell stimulation? One could envisage that regulatory sequences within the U3 region act to increase the transcriptional level of the proviral genome or a gene flanking the provirus, resulting in high level expression

of a viral or cellular gene. Indeed, several studies have demonstrated that transcription enhancement by the MPSV LTR is more efficient than that of the Mo-MuLV LTR in several undifferentiated and differentiated cell types (5, 7–9, and Beck-Engeser, G. et al., manuscript submitted for publication). Two regions of the MPSV LTR appear to be important for this distinctive property of MPSV-derived retroviral vectors: a new SP1 binding site generated by a single point mutation downstream of the direct repeat, and possibly several point mutations clustered at the 5′ end of the direct repeat (M. Grez, manuscript in preparation).

The high incidence (20% of LB3 clones or >17% of E9.D4 clones) at which the stimulation requirement was abrogated excludes the possibility that the site of provirus integration is important, as the infection protocol (MOI <1) was designed to ensure no more than one retroviral copy per genome, and this was confirmed by Southern blot analysis of infected LB3 clones (data not shown). This rules out the contribution of host sequences, but leaves the possibility that viral-encoded proteins may contribute to the altered stimulation requirement.

Two genes, *neo*^{R} and *gag*, are known to be expressed by both neo^R MPSV and neo^R/mos$^-$. However, it is unlikely that they are responsible for the loss of stimulation requirement. An enhanced expression of the gene under G418 selection would result in better survival of infected clones in the replica plating assay. Our data, however, clearly demonstrate that clones survive better when maintained without selective pressure (Fig. 5). Moreover, the lack of a difference in the efficiency of expressed gene transfer (neo^R colonies, Table 1) on infection with vectors with the Mo-MuLV U3 region compared with those infected containing the MPSV U3 region would strongly suggest that the level of expression of the viral transcript is not directly involved in abrogating the indicated growth requirements of these T cell lines, since we and others have demonstrated that differential transcription rates of Mo-MuLV or MPSV vectors usually reflect different efficiencies of recovering G418-resistant colonies (5, 30).

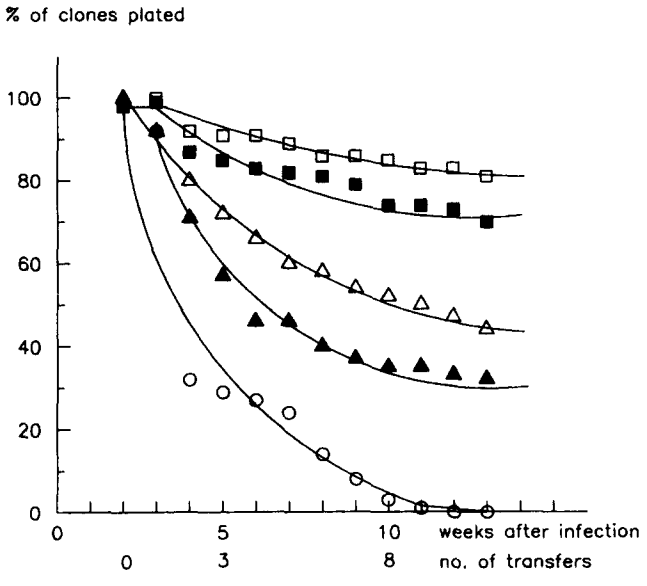

Figure 5. Influence of G418 selection on growth of infected cells. Replica plating assay of MPSV-infected E9.D4 clones. Clones were obtained from primary infection plates and maintained as described in Fig. 3. After the first transfer, positive wells were split and maintained with (*filled symbols*) or without (*open symbols*) G418. Mock-infected E9.D4 clones (○, n = 527); neo^R MPSV-infected E9.D4 clones (△, n = 581); neo^R MPSV mos$^-$-infected E9.D4 clones (□, n = 384).

Immunoprecipitation using anti-p30, anti-p15, and anti-p12 has indicated that a *gag*-related protein of 62–65 kD is translated from the full-length RNA of the MPSV viral genome. Pulse-chase and Western blot analysis of the MPSV *gag* precursors indicated that they are intracellularly stable and do not give rise to mature virion proteins (40). The hypothesis that *gag* precursor proteins interfere with stimulation pathways in infected target cells, however, can probably be ruled out, since infection with the MOV/mos$^-$ construct with the identical coding region does not result in abrogation of the stimulation requirement.

Another transforming mechanism by sequences within the MPSV U3 LTR may involve competition for factors that alter the expression of a cellular gene that is involved in the mitogenic response of activated T cells. Sequence analysis of the unique U3 region has revealed recognition sequences for several transcription factors, including a unique SP1 binding site (9, and M. Grez, manuscript in preparation). However, it is difficult to imagine a mechanism in which a single viral copy per cellular genome could effectively alter the distribution of proteins involved in gene regulation. Factors that regulate transcription and translation by binding RNA are poorly understood, but it cannot be excluded that these may play a role in the MPSV-induced "transformation" of lymphocytes.

We report here a unique mechanism by which the stimulation requirement of T cell lines is abrogated. This is in striking contrast to the long observed involvement of retroviruses in human adult T cell leukemia (41–43), in which autocrine IL-2 stimulation and/or lymphokine expression can be linked to the *trans*-activator protein p40X encoded by the HTLV-I genome. The p40^x (or *tax*) protein can bind to its own viral LTR and thus enhance retroviral transcription, but it binds as well to distinct sequences in the 5′ flanking regions of the IL-2 and IL-2R genes, thus, *trans*-activating gene expression. Moreover, it was demonstrated that mitogen-induced activation of IL-2 and IL-2R genes was mediated via the same binding sites and was coupled with p40^x activation (44). Furthermore, Böhnlein et al. (45) have reported sequence homology between the HTLV-I LTR and the IL-2α receptor gene, both of which are regulated by the same nuclear binding proteins. The MPSV-based vectors, however, contain no known coding region for a putative *trans*-activating protein and do not induce IL-2 expression.

Taken together, the effect of MPSV infection of representative T cell lines does not easily explain the transforming potential of MPSV in vivo. Our results, however, provide evidence that MPSV-infected T cells might have a proliferative advantage over uninfected cells in vivo. These cells may

no longer depend on TCR-specific activation for clonal expansion. More T cells would then be available to respond to antigenic stimulation and secrete lymphokines. The role of the *mos* oncogene in the induction of the myeloproliferative disease remains unclear, but could be important for viral persistence and spread in vivo, e.g., by transformation of connective tissue cells.

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Address correspondence to Wolfram Ostertag, Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse S2, D-2000 Hamburg 20, FRG.

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