Brief Definitive Report

CD2-mediated Autocrine Growth of Herpes Virus Saimiri-transformed Human T Lymphocytes

By Hans-Willi Mittrücker, Ingrid Müller-Fleckenstein,* Bernhard Fleckenstein,* and Bernhard Fleischer

*From the First Department of Medicine, University of Mainz, D-6500 Mainz; and the *Institute for Clinical and Molecular Virology, University of Erlangen-Nürnberg, D-8250 Erlangen, Germany*

Summary

Herpes virus saimiri (HVS) immortalizes T lymphocytes from a variety of primates and causes acute T cell lymphomas and leukemias in nonnatural primate hosts. Here we have analyzed the requirements for growth of three HVS-transformed human T cell lines. The cells expressed the phenotype of activated T cells: two were CD4$^+$, and one was CD8$^+$. All three cells responded to all allogeneic human cell lines tested with enhanced proliferation, production of interleukin 2 (IL-2), and increased expression of the IL-2 receptor. Binding of CD2 to its ligand CD58 was the critical event mediating stimulation because: (a) monoclonal antibodies (mAbs) to CD2 and to CD58, but not to a variety of other surface structures, blocked induced and spontaneous proliferation and IL-2 production; (b) only anti-CD2 mAbs were stimulatory if crosslinked; (c) a nonstimulatory cell was rendered stimulatory by CD58 transfection; and (d) the cells responded specifically to CD58 on sheep red blood cells. Growth of the cells required activation because cyclosporin A and FK506 blocked stimulator cell–induced IL-2 production and proliferation as well as the spontaneous growth of the lines. Antibodies to the IL-2 receptor reduced proliferation of the cells and blocked IL-2 utilization. Taken together, these results show that HVS-transformed T cells proliferate in response to CD2-mediated contact with stimulator cells or with each other in an IL-2-dependent fashion. They suggest that HVS transforms human T cells to an activation-dependent autocrine growth.

Herpes virus saimiri (HVS) is the prototype of the lymphotropic γ-2 herpes viruses (rhadinoviruses). Natural hosts of the virus are the squirrel monkeys (*Saimiri sciureus*). By cocultivation with permissive monolayer cells, HVS can be isolated from PBL >80% of the population, and at least one in 10^6 T cells will yield virus (reviewed in reference 1). The virus persists in T cells for their lifetime. HVS is not pathogenic for squirrel monkeys, even after immunosuppression. In contrast to this benign behavior in its natural host, HVS has a high oncogenic potential in numerous other New World primates (1). It causes acute lymphoproliferative syndromes, leukemias, and lymphomas. After infection, the animals die within a few weeks from rapidly progressing neoplasias. Cell lines can be established from infected animals that initially produce virus in ∼1–10% of the cells, but after prolonged cultivation the ability to produce virus is lost (2). HVS is able to transform T lymphocytes of nonhuman primates as well as human PBL and thymocytes to continuous growth (2, 3). The molecular basis of T cell stimulation and transformation is unknown.

In this study we show that transformation with HVS induces human T cells to respond to cell-cell contact with each other and with other cells leading to IL-2-dependent auto- crine growth. The critical stimulating signal in this contact is given by binding of the CD2 molecule to its natural ligand. These HVS-transformed T cells are the first example of an activation-dependent growth of transformed cells and could be a valuable model for viral oncogenesis.

Materials and Methods

Cells. HVS-transformed human T cell lines were initiated by infection with the 488 strain that belongs to subgroup C of HVS as described (3). Three different lines were used in this study: V20 and V25 are CD4$^+$ subclones of a CD4$^+$ T cell line derived from adult PBMC, CB15 is a CD4$^+$ cell line derived from cord blood lymphocytes, and P1084 is a CD8$^+$ clone derived from thymocytes. These lines had been in culture for 6–12 mo when the experiments described here were performed.

Clones of normal human T cells of CD4$^+$ or CD8$^+$ phenotype were generated and propagated as described (4). Several established tumor cells were used as stimulator cells: the Burkitt's lymphoma Raji and its MHC class II–negative mutant RJ.225 (5); the L428 cells derived from Hodgkin's disease that are HLA class I negative, class II positive, CD11/CD18 negative, and express large amounts of CD58; several different EBV-transformed B cells; and the following mouse cell lines: the mastocytoma P815, the plasmocytoma

Table 1. *Response of HVS-transformed Cells to Allogeneic and Xenogeneic Cells*

Stimulus	Response of V20 cells		Response of P1084 cells	
	Proliferation	IL-2 production	Proliferation	IL-2 production
Medium	1,300	<0.1	7,000	<0.1
Raji	27,600	4.1	26,500	10.2
RJ.225	10,300	2.9	26,300	8.3
L428	20,400	11.5	30,600	11.6
EBV⁺ B cell	17,100	3.2	16,800	4.3
A20/J	3,600	<0.1	8,000	<0.1
A20-D45	3,300	<0.1	7,000	<0.1
A20-P24	17,800	3.6	16,100	5.4
P3X63	5,600	<0.1	10,600	ND
P815	3,000	0.2	11,400	<0.1

V20 cells (CD4⁺) and P8014 (CD8⁺) cells were stimulated with allogeneic or xenogeneic mitomycin C–treated cells, and proliferation (cpm incorporated into responder cells) and IL-2 production (units IL-2/ml culture supernatant) were measured.

P3X63Ag8, the macrophage line P338D1, and B cell lymphoma A20/J, and two derivatives: a DR3-transfected subline (A20-D45) and a CD58-transfected subline (A20-P24; reference 6).

Reagents. The following mAbs were used: three mAbs against CD58 (LFA-3): TS2/9 specific for human CD58 (7), L180/1 specific for the sheep homologue of CD58 (8), and AICD58.3 recognizing both human and sheep CD58. Three mAbs against the SRBC-binding T11-1 epitope of CD2: CB.219 (4), OKT11, and GT2, as well as mAb D66 against CD2R (7, 9, 10). OKT3 against CD3; BMA031 against the TCR α/β; MT151, MT321, and BMA041 against CD4; MT 61 against CD5; and BMA081 against CD8 (7). Also used were anti-Tac (11) against the 55-kD chain of the IL-2 receptor, TS1/22 against CD11a, 7F7 against the intercellular adhesion molecule 1 CD54 (10), W6/32 against HLA class I antigens, L243 against HLA-DR, CLB.CD28 against CD28 (10), and BB.1 against the CD28 ligand B7 (12).

Assays for Proliferative Activity and IL-2 Production. Proliferative responses were measured in triplicates of 200 μl containing 2–3 × 10⁴ T cells as responders and 3 × 10⁴ mitomycin C–treated tumor cells as stimulator cells (4). Assays were pulsed with [³H]thymidine after 24 h for 16 h. For determination of IL-2 production, supernatants of such cultures were harvested after 24 h and assayed for IL-2 using CTLL-2 cells.

Results

Response of HVS-transformed T Cells to Allogeneic Cells. The three cell lines expressed the phenotype of activated T cells. They were CD2⁺, CD3⁺, TCR α/β⁺, and expressed the IL-2 receptor (p55, CD25), CD26, CD58, and HLA class II molecules. The CD4⁺ cell lines were CD28⁺. The growth of the CD4⁺ cells slowed down in the absence of added IL-2 but the cells survived several weeks, whereas the CD8⁺ cell line stopped growing when IL-2 was removed from the culture medium and died within a few days. The cells grew best at high densities, and cell growth decreased or stopped if the cells were seeded at low density.

The HVS-transformed cells responded to allogeneic human stimulator cells alone with increased proliferation and IL-2 production (Table 1). Spontaneously, there was little or no secretion of IL-2 detectable, but strong IL-2 production was found in the presence of all human cell lines tested. It is evident from the phenotype of the stimulator cells used that this stimulatory activity for both CD4⁺ and CD8⁺ cells

Table 2. *Stimulation of IL-2 Production in HVS-transformed T Cells*

mAb added	Specificity	IL-2 production by CB15 cells with:	
		L428	A20/J
None		6.2	0.1
CB.219	CD2 (IgG2b)	<0.1	3.5
OKT3	CD3 (IgG2a)	6.4	0.2
BMA031	TCRαβ (IgG2b)	7.1	0.1
BMA041	CD4 (IgM)	5.6	0.1
M-T151	CD4 (IgG2a)	4.9	<0.1
M-T321	CD4 (IgG1)	6.9	0.1
M-T61	CD5 (IgG1)	6.0	0.1
CLB.CD28	CD28 (IgG1)	6.7	0.1
TS1/22	CD11a (IgG1)	7.3	<0.1
TS2/9	CD58 (IgG1)	<0.1	<0.1
W6/32	class I (IgG2a)	8.4	0.1
L243	HLA-DR (IgG2a)	7.0	0.1

CB15 cells stimulated with L428 or A20/J cells in the presence of the mAb indicated (1 μg/ml or 1:1,000 dilution of ascitic fluid). Supernatants were assayed for IL-2 after 24 h. Data are units IL-2/ml.
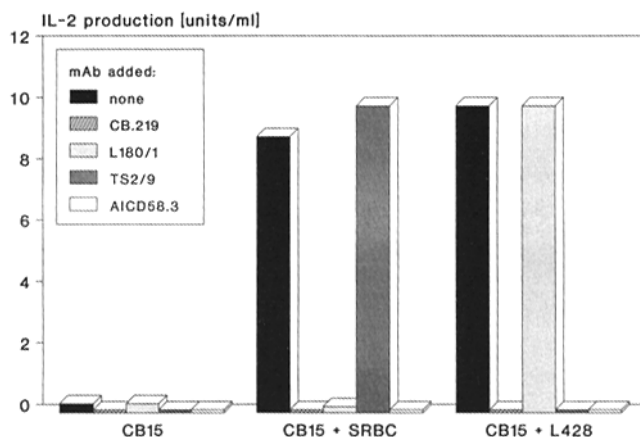
Figure 1. Response to SRBC of HVS-transformed cells is especially inhibited by mAbs to sheep CD58. CB15 cells were incubated in flat-bottomed wells with SRBC (0.25%; vol/ml) or L428 cells and mAb CB.219 (anti-CD2), L180/1 (anti-sheep CD58), TS2/9 (anti-human CD58), or AICD58.3 (anti-sheep and -human CD58).

Table 3. *Growth and Expression of the Activated Phenotype Require Activation*

| Additions | Proliferation* | | Production of IL-2[‡] | Expression of:[§] | |
	−IL-2	+IL-2		CD25	HLA-DR
Medium	6,000	13,600	<0.1	480	580
CyA	200	6,300	<0.1	350	430
Raji	32,100	30,300	14	550	510
Raji + CyA	2,400	6,900	<0.1	370	440

V25 cells were incubated with various additions for 2 d, whereafter the cells were pulsed with [³H]thymidine for 24 h. IL-2 production was assayed in parallel cultures after 24 h of incubation. The expression of CD25 and HLA-DR molecules was determined after incubation of 5×10^5 cells/ml for 4 d with the additions indicated.
* cpm incorporated by V25 cells
‡ Units IL-2/ml supernatant
§ Mean channel fluorescence intensity (FACScan®). Staining with a negative control mAb gave an intensity of 100, staining with OKT3 mAb gave an intensity of 660–680 irrespective of treatment.

was not dependent on the presence of HLA class I or class II molecules. Fixation with glutaraldehyde did not decrease the stimulating capacities of allogeneic cells, indicating that a secreted product of the stimulating cells did not play a role (not shown). Several murine cell lines, some of them shown in Table 1, did not induce enhanced proliferation and IL-2 secretion or were only very weak stimulators. Interestingly, the CD58-transfected A20-P24 cells, but neither the parental A20/J cells nor the HLA-DR3-transfected A20/J cells, had stimulatory capacity for all three HVS-transformed T cells.

Cell Surface Molecules Involved in Stimulation. To see which cell surface structures were involved in the stimulating reaction, we used several mAbs to block triggering of proliferation and IL-2 production. Table 2 shows a typical experiment with CB15 (CD4+) responder cells and L428 and A20/J cells as stimulators. The only mAbs that completely inhibited the response to L428 cells were those to CD2 and its ligand CD58. mAbs to CD3 and the TCR did not block stimulation significantly, and several anti-CD4 mAbs and TS1/22 to CD11a only sometimes showed slight inhibition. Although not shown here, the 7F7 mAb to CD54, the BB.1 mAb to the CD28 ligand B7, and several anti–class II mAbs did not inhibit. There was also no influence of mAb D66 directed against a restricted stimulating epitope on CD2 not involved in SRBC binding (not shown). Noteworthy, only antibodies to CD2 and CD58 blocked spontaneous IL-2 production and reduced spontaneous proliferation (data not shown).

The three anti-CD2 mAbs directed against the T11-1 epitope were able to induce IL-2 production (as shown for CB.219 in Table 2) and enhanced proliferation (not shown) if cross-linked by the nonstimulating A20/J cells that are Fc receptor positive. In contrast, all other antibodies, including D66, OKT3, BMA031, and CLB.CD28, did not induce significant IL-2 secretion.

To directly assess the role of CD58 in this stimulation, we used SRBC as stimulators and two mAbs that discriminate between human and sheep CD58. Indeed, incubation of HVS-

transformed cells with SRBC stimulated IL-2 production (Fig. 1) and enhanced proliferation (not shown). This stimulation was completely abrogated by anti-CD2 mAbs and by the mAb L180/1 to sheep CD58 but not by TS2/9 to the CD58 expressed by the responder cells.

IL-2 Production and IL-2 Receptor Expression Require Activation. To obtain further evidence that proliferation and IL-2 production were triggered via pathways involved in normal T cell activation, we used the immunosuppressants cyclosporin A (CyA) and FK506, which interfere with T cell signal transduction. Both compounds inhibited IL-2 production and proliferation induced by allogenic cells in CD4+ and CD8+ HVS-transformed cells. This is shown in Table 3 for CyA and the response of V25 cells. The concentrations required were the same that were effective in normal human T cell clones.

Moreover, CyA treatment could downregulate the IL-2 receptor on HVS-transformed cells. When the cells were incubated for 4 d in 1 μg/ml CyA, they became smaller and resembled resting T cells. As shown in Table 3, the expression of the 55-kD chain of the IL-2 receptor (CD25) and of HLA class II molecules on these cells decreased. If untreated HVS-transformed cells were incubated with Raji cells, an enhancement of CD25 expression was seen that could be blocked by CyA (Table 3). Noteworthy also, the response to IL-2 was reduced by CyA. In addition, in the presence of CyA, V20, V25, and P1084 cells stopped growing even in the presence of IL-2. Identical findings were made with FK506 at 100-fold lower concentrations. Taken together, this indicates that the activated phenotype of the cells was due to the permanent reception of activation signals.

Evidence for Autocrine Growth. Because production of and response to IL-2 appeared to depend on permanent stimulation, we investigated if HVS-transformed T cells would grow in an IL-2-dependent autocrine fashion. In the absence of stimu-

Table 4. *Inhibition of Proliferation by a mAb against the IL-2 Receptor*

| | Proliferative response of: | | | | | |
| | P1084 cells | | | V20 cells | | |
Stimulus	Medium	Anti-Tac	Inhibition	Medium	Anti-Tac	Inhibition
Medium	18,000	11,000	39%	1,700	1,200	30%
L428 cells	40,000	42,000	0%	37,500	37,300	0%
IL-2	25,000	18,000	28%	25,200	3,600	86%

HVS-transformed cells were incubated with L428 cells or 20 U/ml of rIL-2, and their proliferation was assayed after 48 h. Data are given as cpm [^{3}H]thymidine incorporated and percent inhibition by anti-Tac.

lator cells, HVS-transformed cells proliferated but IL-2 was not or in only low amounts detectable in the supernatant. Addition of exogenous IL-2 enhanced proliferation and anti-Tac mAb inhibited spontaneous and IL-2-induced but not stimulator cell–induced proliferation of V20, V25, and the CD8$^+$ cells in short-term assays. Such an experiment with the P1084 CD8$^+$ and the V20 CD4$^+$ cell lines is shown in Table 4. CB15 cells were less sensitive to anti-Tac (not shown). Furthermore, anti-Tac mAb was able to increase the concentration of IL-2 in the supernatant of all three cell lines, with both CD4$^+$ and CD8$^+$, indicating that anti-Tac blocked utilization of IL-2 (data not shown).

Discussion

This report describes three independent lines of HSV-transformed human T lymphocytes. The three cell lines express the phenotype of activated T cells and possess the functional capacities of normal T lymphocytes: a proliferative response, lymphokine secretion, and induction of IL-2 receptors.

A common characteristic of these cells is their response to allogeneic stimulator cells. Apparently, any allogeneic cell, regardless of HLA class I or II expression, can stimulate HVS-transformed cells. This argues against the recognition of a superantigen or of a promiscuous peptide on the stimulator cells. Rather, the stimulating signal appears to be given by binding of the CD2 molecule to its natural ligand. The evidence for this notion is provided by the selective inhibition of stimulation by anti-CD2 and anti-CD58 mAbs, the potent stimulatory activity of mAbs directed against the ligand binding site of CD2, and, most notably, the response to SRBC and to CD58 transfectants. The response to SRBC suggests that the CD58 molecules is stimulatory in the absence of other ligands for T cell surface structures. That several mouse cells were not or only weakly stimulatory is probably due to the inefficient interaction of human CD2 with mouse CD58 (6), in contrast to human CD11a/CD18, which binds mouse intercellular adhesion molecule 1 efficiently (13). HVS-transformed cells are the only example showing that binding of CD2 to its natural ligand alone is sufficient to activate a T cell. mAbs to restricted sites of the CD2 molecule, such as D66 or T11$_3$, have been shown to activate T cells if com-

bined with mAbs to the ligand binding site or the ligand itself (9, 14). However, the general relevance of this type of stimulation is still unclear. Such a mAb (D66) was not stimulatory for HVS-transformed cells, but rather inhibited the activation via the anti-CD2 mAb CB.219 (not shown). Surprisingly, mAbs to CD3 or to the TCR did not or only weakly induced IL-2 production and enhanced proliferation with Fc receptor–bearing accessory cells and did not inhibit the response to allogeneic cells. This is in striking contrast to the behavior of normal human T cells, which are stimulated by mAbs to the CD3/TCR complex but not by the anti-CD2 mAbs. This could indicate that CD2 constitutes an independent pathway in the herpes virus–transformed cells that is uncoupled from TCR/CD3 signaling.

Our data strongly suggest that this interaction of CD2 with CD58 during mutual cell-cell contact is the signal leading to autocrine growth of HVS-transformed cells. Most notably, incubation of the cells with anti-CD2 or anti-CD58 mAbs reduced growth of the cells. The findings with CyA and FK506 also support the notion that the growth of the HVS-transformed cells is due to the permanent reception of activating signals during cell-cell contact. These compounds inhibited IL-2 production as well as growth of HVS-transformed cells. The experiments with anti-Tac suggest that IL-2-mediated growth was the principle governing the growth of the V20 and the CD8$^+$ cells. That anti-Tac mAb did not inhibit optimally stimulated cells is also found with some normal human T cell clones (data not shown) and could be due to an excess production and short-range action of IL-2 (11).

In spite of these common mechanisms of responses the three cell lines had different growth characteristics. The CD4$^+$ V20 and V25 subclones and the CD8$^+$ P1084 line had a low spontaneous background proliferation and responded with strongly enhanced proliferation to stimulator cells. These cells required exogenous IL-2 for growth and survival if they were cultured at low density. In contrast, the CB15 cells had a high background proliferation that usually was >50% of the maximal response. This line grew in the absence of exogenous IL-2 and, if supplied with IL-2, was only poorly inhibited in its growth by CyA. Thus, the CB15 cells appear to be more autonomous than the other cell lines, possibly at an advanced stage of transformation. These properties of the cells

were stable within the observation period. It is noteworthy that the CB15 cell line had been initially established in the absence of exogenous IL-2.

Autocrine growth factor production is a recognized mechanism of autonomous growth (15). So far, however, in the reported examples the respective growth factor is produced constitutively. Transfection of a growth factor into factor-dependent cells leads to autonomous proliferation and tumorigenicity in vivo (15). The HVS-transformed cells are the first example of an autocrine growth of transformed cells with an activation signal–dependent growth factor production. Only two other cells have been reported that show a similar behavior. A human T cell leukemia cell was described that

expressed IL-2 receptors, produced IL-2, and proliferated autonomously at high cell densities only (16). Growth of the cells was inhibited by CyA (17). In addition, a recent report showed that the IL-4-dependent growth of RadLV-transformed murine T cells is sensitive to CyA (18). This could indicate that similar, although still uncharacterized, activation-dependent mechanisms are operating in these cells.

In summary, our results suggest that HVS-transformed T cells grow via an autocrine mechanism, in response to activation signals given by their CD2 molecules during mutual cell-cell contact. These cells are the first example of an activation-dependent autocrine growth and will be a promising model for viral oncogenesis.

References

1. Fleckenstein, B., and R.C. Desrosiers. 1982. Herpesvirus saimiri and Herpesvirus ateles. In The Herpesviruses I. B. Roizman, editor. Plenum Publishing Corporation, New York. 253–331.
2. Schirm, S., I. Müller, R.C. Desrosiers, and B. Fleckenstein. 1984. Herpesvirus saimiri DNA in a lymphoid cell line established by in vitro transformation. *J. Virol.* 49:938.
3. Biesinger, B., I. Müller-Fleckenstein, S. Simmer, G. Lang, S. Wittmann, E. Platzer, R.C. Desrosiers, and B. Fleckenstein. 1992. Stable growth transformation of human T lymphocytes by herpesvirus saimiri. *Proc. Natl. Acad. Sci. USA.* 89:3116.
4. Fleischer, B. 1987. A novel pathway of human T lymphocyte activation via a 103 kd T cell activation antigen. *J. Immunol.* 138:1346.
5. Acolla, R.S. 1983. Human B cell variants immunoselected against a single Ia antigen subset have lost expression of several subsets. *J. Exp. Med.* 157:1053.
6. Moingeon, P., H.-C. Chang, B.P. Wallner, C. Stebins, A.Z. Frey, and E.L. Reinherz. 1989. CD2-mediated adhesion facilitates T lymphocyte antigen recognition function. *Nature (Lond.).* 339:312.
7. McMichael, A.J., Ed. 1987. Leukocyte Typing III. Oxford University Press, Oxford. 1048 pp.
8. Hünig, T. 1985. The cell surface molecule recognized by the erythrocyte receptor of T lymphocytes. *J. Exp. Med.* 162:890.
9. Brottier, P., L. Boumsell, C. Gelin, and A. Bernard. 1985. T cell activation via CD2 (T, gp50) molecules: accessory cells are required to trigger T cell activation via CD2-D66 plus CD2-9.6/T11(1) epitopes. *J. Immunol.* 135:1624.
10. Knapp, W., Ed. 1989. Leukocyte Typing IV. Oxford University Press, Oxford. 1182 pp.
11. Depper, J.M., W.J. Leonard, R.J. Robb, T.A. Waldmann, and W.C. Greene. 1983. Blockade of the IL-2 receptor by anti-Tac: inhibition of human lymphocyte activation. *J. Immunol.* 131:690.
12. Yokochi, T., R.D. Holly, and E.A. Clark. 1982. B lymphoblast antigen (BB.1) expressed on Epstein-Barr virus activated B cell blasts, B lymphoid lines, and Burkitt's lymphomas. *J. Immunol.* 128:823.
13. Johnston, S.C., M.L. Dustin, M.L. Hibbs, and T.A. Springer. 1990. On the species specificity of the interaction of LFA-1 with intercellular adhesin molecules. *J. Immunol.* 145:1183.
14. Hünig, T., G. Tiefenthaler, K.-H. Meyer zum Büschenfelde, and S.C. Meuer. 1987. Alternative pathway activation of T cells by binding of CD2 to its cell-surface ligand. *Nature (Lond.).* 326:298.
15. Lang, R.A., and A.W. Burgess. 1990. Autocrine growth factors and tumorigenic transformation. *Immunol. Today.* 11:244.
16. Duprez, V., G. Lenoir, and A. Dautry-Varsat. 1985. Autocrine growth stimulation of a human T cell lymphoma line by interleukin 2. *Proc. Natl. Acad. Sci. USA.* 82:6932.
17. Dautry-Varsat, A., A. Hemar, V. Cornet, and V. Duprez. 1988. Autocrine growth of a human T cell line is inhibited by cyclosporin A. *Blood.* 72:588.
18. Yefenof, E., G. Abboud, S. Epszteyn, and E. Vitetta. 1992. Treatment of premalignancy: prevention of lymphoma in radiation leukemia virus-inoculated mice by cyclosporin A and immunotoxin. *Proc. Natl. Acad. Sci. USA.* 89:728.