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Transformation of human T-cell clones by Herpesvirus saimiri: Intact antigen recognition by autonomously growing myelin basic protein-specific T cells

(autoimmunity/autoimmune T cell/immunotherapy/multiple sclerosis/T-cell receptor)

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ABSTRACT **Herpesvirus saimiri has recently been shown to immortalize human T cells. It was unknown, however, whether Herpesvirus saimiri transformation affects T-cell receptor (TCR) expression and signal transduction. In the present study, we have transformed CD4$^+$ human T-cell clones specific for human myelin basic protein. The transformed T cells were grown in interleukin 2 and divided in the absence of antigen and antigen-presenting cells. They retained the membrane phenotype of activated T cells and secreted the cytokines interferon γ and lymphotoxin, but interleukin 4 was not detected. Further, the transformed T cells continued to express the original TCR as demonstrated by TCR variable-region-Vβ-specific monoclonal antibodies and TCR sequencing. Antigen-specific recognition and signal transduction by the TCR were demonstrated by myelin-basic-protein-induced HLA-DR-restricted secretion of interferon γ and lymphotoxin and by myelin-basic-protein-specific proliferation. Antigen specificity and reactivity have been maintained for >1 year after transformation. Transformation with Herpesvirus saimiri now allows the production of virtually unlimited numbers of (auto)antigen-specific T cells expressing functional TCR and a stable membrane phenotype. This technology will facilitate studies of the pathogenesis of putative autoimmune diseases, such as multiple sclerosis, and may be of help in TCR-targeted immunotherapy.**

Autoantigen-specific autoaggressive T-cell clones have become indispensable tools to study the cellular and molecular mechanisms of autoimmunity. For example, studies using myelin-specific T-cell lines have provided insights into the details of demyelinating autoimmune responses and into the general organization of intracerebral immune reactivity and seem to provide a basis for additional therapeutic strategies (1). Once isolated from the donor organism, autoimmune T cells can be cultured for a few weeks in interleukin (IL) 2-containing medium in the absence of antigen. For prolonged culture, the cells must be restimulated at regular intervals with antigen and antigen-presenting cells (APCs) (2, 3). Even under optimal culture conditions, most human and rodent T-cell lines have finite life spans. This and the requirement of fresh human APCs form major obstacles limiting the duration and dimension of human T-cell studies.

These problems could be overcome by virus transformation of antigen-specific T cells, in analogy to Epstein–Barr virus transformation of B cells. To be useful for broad application, the ideal T-cell-transforming agent should lead to permanent APC- and antigen-independent growth, while conserving the essential features of the nontransformed parental T cells. We show here that Herpesvirus saimiri (HVS), a lymphotropic γ-2 herpesvirus (4), fulfills most, if not all, of these criteria. Using cells specific for myelin basic protein (MBP), one of the best characterized experimental and human autoantigens and a probable target structure in the pathogenesis of multiple sclerosis (5–7), we demonstrate that HVS can transform antigen-specific CD4$^+$ human T-cell clones to permanent growth. The transformed T cells express their antigen receptor in unaltered form and recognize their specific antigen as documented by antigen-induced major histocompatibility complex (MHC)-restricted proliferation and cytokine secretion.

MATERIALS AND METHODS

T-Cell Clones. Human CD4$^+$ T-cell clones specific for human MBP (isolated according to ref. 8) were generated from the blood of patients with multiple sclerosis as described (9). The T-cell clones were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, penicillin (10^2 units/ml), streptomycin (100 μg/ml) (GIBCO/BRL), 5% (vol/vol) heat-inactivated AB$^+$ human serum from local volunteers, and human recombinant IL-2 [50 units/ml; kindly provided by F. Sinigaglia, Hoffmann–La Roche, and by Eurocetus (Amsterdam)] at 37°C in 5% CO_2/95% air. Restimulation was performed every 2 weeks by presenting MBP (30 μg/ml) on irradiated (50 Gy) autologous peripheral blood mononuclear cells (PBMCs). Antigen specificity was determined by [^{3}H]thymidine (Amersham) incorporation. Antigen fine specificity and MHC restriction of the T-cell clones were established using a set of 17 overlapping synthetic peptides covering the entire length of human MBP and HLA-DR-transfected mouse L cells as APCs (10).

L-Cell Transfectants. L-cell transfectants expressing HLA-DR1 (DRA/DRB1*0101) (9) and the two products of the DR2Dw2 haplotype, DRA/DRB1*1501 and DRA/DRB5*0101 (11), were kindly provided by D. Altman (Transplantation Biology Section, Harrow, U.K.) and J. Trowsdale (Imperial Cancer Research Fund, London). Mouse LTK$^-$ cells were used as controls.

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Abbreviations: APC, antigen-presenting cell; HVS, Herpesvirus saimiri; IFN, interferon; IL, interleukin; LFA, leukocyte function-associated antigen; mAb, monoclonal antibody; MBP, myelin basic protein; MHC, major histocompatibility complex; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cell; TCR, T-cell receptor; C, constant; V, variable.

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Transformation of T Lymphocytes with HVS. T cells were restimulated with irradiated PBMCs and MBP. Three days after restimulation, exogenous human recombinant IL-2 (50 units/ml; Boehringer Mannheim) was added. The cells were seeded at a density of 10^6 cells per ml and inoculated with 10^5 tissue culture ID_{50} units of HVS subgroup C strain 488 (12, 13). When using a few million vital cells for infection, the efficiency rate of transformation reaches 100% (for review, see ref. 14). Production of infectious virus was monitored by cocultivation of lymphocytes with permissive owl monkey kidney cells. Viral DNA was detected by a gel electrophoresis technique (15).

Determination of TCR Gene Usage. Total RNA was prepared from 2 to 3 × 10^6 T cells as described (16). Oligo(dT)-primed double-stranded cDNA was synthesized from 2 to 3 μg of total RNA using Moloney murine leukemia virus-derived reverse transcriptase (GIBCO/BRL) essentially as described by the supplier. Blunt-ended cDNA was then circularized during a 5-h incubation at room temperature with T4 DNA ligase (GIBCO/BRL) in a volume of 20 μl. The ligated material (1 μl) was used as a template for an inverse polymerase chain reaction (PCR) basically as described (17). Briefly, α- and β-chain amplifications were performed separately in 50-μl reaction mixtures containing 1× PCR buffer (17), 1 unit of *Taq* polymerase (Boehringer Mannheim), constant (C) region primers for α or β chains (0.5 μM), and all four dNTPs (each at 200 μM). The PCR primers used are as follows: Cα forward primer, 5′-CAA TGG ATC CTT GTC ACT GGA TTT AGA GTC; Cα inverse primer, 5′-GGA ATT CCT GCT ATG CTG TGT GTC TGG; Cβ forward primer, 5′-GGG TCG ACG GTG TGG GAG ATC TCT GC; Cβ inverse primer, 5′-GGA ATT CTG TCT GCC ACC ATC CTC TAT GAG. Cα primers contain *Eco*RI and *Bam*HI; Cβ primers contain *Eco*RI and *Bgl* II restriction sites. PCR was done for 35 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min). PCR products were purified by phenol extraction, precipitated with ethanol, and excessively digested with the restriction endonucleases mentioned above. Fragments of expected sizes for the cDNAs were recovered from a preparative low-melting-point agarose gel and ligated into M13mp18 previously cut with *Eco*RI and *Bam*HI. Single plaques were picked and grown up, and recombinant phage DNA was purified for DNA sequence determination. For each T-cell clone at least 12 isolates were sequenced by the chain-termination method (18) using the Pharmacia T7 sequencing kit.

Proliferation Tests. Approximately 3 × 10^4 T cells were incubated in flat-bottom 96-well microwells with 5 × 10^4 HLA-DR-transfected mouse L cells (treated with mitomycin), or in U-shaped 96-well plates with 2 × 10^5 irradiated PBMCs in the presence or absence of antigen (final concentration: MBP, 30 μg/ml; peptide, 10 μg/ml). [^{3}H]Thymidine (0.22 μCi per well; 1 Ci = 37 GBq; Amersham) was added after 48 h. Cells were harvested 18 h later. [^{3}H]Thymidine incorporation was measured with a direct counting system (Matrix TM 96, Direct Beta Counter, Packard), yielding ≈20% of the cpm obtained with liquid scintillation systems. The stimulation indices, however, are essentially the same.

Cytokine Measurements. Approximately 3 × 10^4 T cells were restimulated as described above in a total volume of 110 μl per well. Supernatants were collected after 24 h for IL-4 and after 48 h for interferon (IFN) γ and lymphotoxin measurements. The supernatants were pooled, centrifuged (1000 rpm, 4°C), and stored in appropriate aliquots at −70°C. All cytokines were measured by ELISA.

IFN-γ ELISA. The polyclonal protein A-purified rabbit anti-human IFN-γ serum was a generous gift of Knoll AG, Ludwigshafen, Germany. This polyclonal antibody was used for coating and after biotinylation for detection. Human recombinant IFN-γ (Hoffmann–La Roche) was used as a standard. The reaction products were visualized using streptavidin-coupled peroxidase and substrate (Pierce). The reaction was stopped with H_2SO_4 and extinction was read at 492 nm.

Lymphotoxin ELISA. The reagents [anti-lymphotoxin monoclonal antibody (mAb) 9B9, polyclonal anti-lymphotoxin antiserum, and human recombinant lymphotoxin] were a generous gift of Knoll AG. The ELISA was performed basically as described above using the mAb for coating and the biotinylated polyclonal antibody for detection.

IL-4 ELISA. IL-4 was detected with a commercially available kit (Biermannn, Bad Nauheim, Germany).

mAbs for Inhibition Experiments and Flow Cytometry. Hybridomas secreting anti-CD2 (TS2/18.1.1), anti-CD3 (OKT3), anti-CD4 (OKT4), anti-CD8 (OKT8), anti-HLA-DR (L243), anti-leukocyte function-associated antigen (LFA)-1α (TS1/22.1.1.13), anti-LFA-1β (TS1/18.1.2.11.4), anti-LFA-3 (TS2/9.1.1.4.3), and anti-IL-2 receptor (CD25) (7G7B6) mAbs were obtained from American Type Culture Collection. IgG1 and IgG2a+b isotype controls were purchased from Becton-Dickinson. Anti-intercellular adhesion molecule-1 (GP-89-14, IgG2b) was provided by J. Johnson (Dept. of Immunology, Munich). Anti-variable (V) region Vβ8 mAb, anti-Vβ12 mAb, and anti-T-cell receptor (TCR) α/β mAbs were from T-Cell Sciences (Cambridge, MA), and anti-Vβ 13.3 mAb was from Dianova (Hamburg, Germany).

Antibody Inhibition Experiments. For inhibition of spontaneous proliferation and cytokine secretion, mAbs against CD2 (LFA-2) and CD58 (LFA-3) were purified by protein A chromatography from culture supernatants containing 10% fetal calf serum. The eluted immunoglobulin was dialyzed overnight against phosphate-buffered saline (PBS), and the protein content was determined with the biuret method. Purified mAbs were used at a final concentration of 2 μg/ml.

Flow Cytometry. T cells were washed once, seeded at a density of 10^5 cells per well in V-shaped 96-well plates, incubated with the indicated mAb or matched isotype controls for 45 min at 4°C in washing buffer (PBS containing 2% heat-inactivated fetal calf serum and 2% human AB^+ serum), washed three times, and then incubated in a 1:150 dilution of fluorescein isothiocyanate-conjugated $F(ab)_2$ fragment goat anti-mouse immunoglobulin (Dianova) for 30 min at 4°C. After washing, the cells were fixed with 1% paraformaldehyde/PBS and analyzed by a fluorescence-activated cell sorter (Becton Dickinson).

RESULTS

Three MBP-specific T-cell clones (CABP6, SSBP8, and CFBP2/6) were generated from the blood of three patients with multiple sclerosis. The cells were expanded *in vitro* by restimulation with HLA-DR-compatible PBMCs, antigen, and exogenous IL-2. The phenotype of all T-cell clones was $CD2^+$, $CD3^+$, $CD4^+$, $CD8^-$, $TCR\alpha/\beta^+$, $HLA\text{-}DR^+$, IL-2 $receptor^+$ (CD25), $LFA\text{-}1\alpha^+$ (CD11a), $LFA\text{-}1\beta^+$ (CD18), $LFA\text{-}3^+$ (CD58), and intercellular adhesion molecule 1^+ (CD54).

The homogeneous staining pattern with one anti-TCR Vβ mAb (Fig. 1 *Left*) suggested that CABP6, SSBP8, and CFBP2/6 are monoclonal. For SSBP8, clonality was demonstrated formally by sequencing of the TCR (ref. 10 and see below).

The three T-cell clones showed a proliferative response to human MBP in the context of three HLA-DR restriction molecules (Table 1). After challenge with MBP and irradiated HLA-DR-compatible PBMCs or HLA-DR-transfected L cells, the T-cell clones produced lymphotoxin and IFN-γ

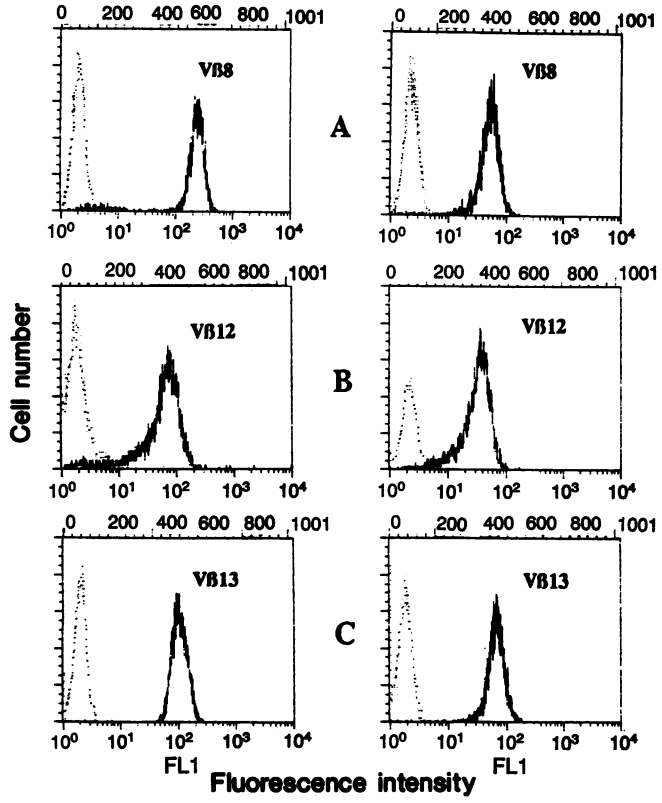

FIG. 1. TCR Vβ expression of nontransformed (*Left*) and HVS-transformed (*Right*) T-cell clones. Three MBP-specific T-cell clones were analyzed. (*A*) CABP6. (*B*) CFBP2/6. (*C*) SSBP8. An isotype-matched control mAb was included in each experiment (dotted line).

(Table 2). In contrast, the T-cell clones produced either no or only a minute amount of IL-4.

All three MBP-specific T-cell clones were transformed with HVS in the first attempt. After transformation, the cells divided for up to 8 weeks in the complete absence of exogenous IL-2. Proliferation was increased in the presence of IL-2 (100 units/ml). For long-term propagation, we kept the T cells in IL-2-containing medium. Under these conditions, the cell number increased by a factor of 2–4 per week of culture.

At various times after transformation, the cultures were screened for the production of infectious virus by cocultivation of the transformed T-cell clones with permissive owl monkey kidney cells. Virus production was not observed later than 3 weeks after infection. All of the transformed T-cell clones contained episomally persisting viral DNA, but no linear virion DNA. None of the clones produced detectable amounts of infectious virus.

The HVS-transformed T-cell clones (CABP6T, SSBP8T, and CFBP2/6T) had the same membrane phenotype as the original nontransformed clones. Further, all transformed clones continued to express the original TCR, as demonstrated by staining with Vβ-specific mAbs (Fig. 1 *Right*). We established the complete sequences of the TCR α and β chain of the T-cell clone SSBP8 [for TCR α chain, Vα23.1, Jα1 (where J is joining region), and the nucleotide sequence GAG GGC GCC GGA at the V–J junction; for TCR β chain, see ref. 10]. The nomenclature of Vα and Jα is from ref. 17 and of Vβ is from ref. 19. The sequences were then compared to the TCR sequences of the corresponding HVS-transformed clone SSBP8T. Both the TCR α and β chains were completely identical between the original and the HVS-transformed clone.

To test for the functional integrity of the TCR expressed by the HVS-transformed clones, the cells were cultured in the presence of HLA-DR-compatible APCs (PBMCs or HLA-DR-transfected L-cells) with or without the specific antigen MBP. When PBMCs were used as APCs, the transformed clones showed no evidence of antigen-specific proliferation (Table 1), but antigen-induced enhancement of cytokine production (Table 2). When HLA-DR-transfected L cells were used as APCs, the transformed clones showed antigen-specific HLA-DR-restricted proliferation (Table 1) and antigen-induced secretion of lymphotoxin and IFN-γ (Table 2). With L cells, but not PBMCs, as APCs, both antigen-specific proliferation and antigen-induced secretion of IFN-γ were more pronounced when the background proliferation and

Table 1. Proliferative responses of transformed and nontransformed MBP-specific T-cell clones in the presence of various APCs

		[^{3}H]Thymidine uptake, cpm			
				DR-transfected L cells	
Clone	MBP	PBMCs	LTK$^-$ cells	APCs	Radioactivity
CABP6	−	18 ± 4	80 ± 7†	DRB1*0101	79 ± 13†
	+	3,620 ± 581	145 ± 30†		7,522 ± 262†
CABP6T	−	3,169 ± 227	758 ± 530		904 ± 611
	+	2,619 ± 8	502 ± 666		2,075 ± 290
SSBP8	−	31 ± 5	6 ± 1	DRB5*0101	151 ± 4
	+	3,841 ± 113	5 ± 3		1,103 ± 96
SSBP8T	−	10,266 ± 1684	4211 ± 505		2,623 ± 786
	+	10,277 ± 1895	6251 ± 786		10,113 ± 1388
CFBP2/6	−	40 ± 17	237 ± 11	DRB1*1501	849 ± 111
	+	5,941 ± 105	294 ± 32		8,927 ± 304
CFBP2/6T	−	9,916 ± 2243	363 ± 274		221 ± 337
	+	5,823 ± 383	614 ± 424		3,449 ± 157

Three MBP-specific T-cell clones (CFBP2/6, CABP6, and SSBP8) were transformed with HVS. The transformed clones are labeled CFBP2/6T, CABP6T, and SSBP8T. Five types of APCs were used—namely, HLA-DRA/B1*0101-, HLA-DRA/B5*0101-, and HLA-DRA/B1*1501-transfected L cells, HLA-DR-compatible PBMCs, and LTK$^-$ cells. The assays with HVS-transformed T-cell clones and DR-transfected L cells or LTK$^-$ cells were performed in the presence of mAb against CD2 (2 μg/ml). CFBP2/6 recognizes residues 61–82 of human MBP in the context of DRA/DRB1*1501; SSBP8 recognizes residues 86–105 in the context of DRA/DRB5*0101; CABP6 recognizes an MBP epitope not represented in the peptide panel (10) or another brain-derived protein in the context of DRA/DRB1*0101 (data not shown). Values are expressed as cpm (mean ± SD).

†Measured by liquid scintillation counting.

Table 2. Cytokine production by transformed and nontransformed T-cell clones

T-cell clone	APC	Lymphotoxin, pg/ml		IFN-γ, pg/ml		IL-4, pg/ml	
		No antigen	MBP	No antigen	MBP	No antigen	MBP
CABP6	PBMCs	0	469	0	14,080	0	158
CABP6T	PBMCs	186	231	18,400	37,800	0	0
	DRB1*0101 cells†	0	715	510	14,860	0	0
SSBP8	PBMCs	104	2,301	0	13,140	ND	ND
SSBP8T	PBMCs	23,344	46,720	19,200	46,000	0	0
	DRB5*0101 cells†	1,668	23,360	4,680	32,560	0	0
CFBP2-6	PBMCs	0	225	140	6,860	0	0
CFBP2-6T	PBMCs	466	1,062	12,320	41,800	0	0
	DRB1*1501 cells†	0	413	3,200	12,100	0	0

Experiments with HVS-transformed T-cell clones and L cells were performed in the presence of mAb against CD2 (2 μg/ml).
†L cells transfected with HLA-DRA and the indicated HLA-DRB gene.

production of IFN-γ were reduced by adding mAbs against CD2, LFA-3 (CD58), or both (Figs. 2 and 3). Antigen specificity could be observed for at least 1 year after transformation.

DISCUSSION

This study has established that antigen-specific human T-cell clones can be transformed to stable growth by transformation with HVS subgroup C strain 488. The transformed T-cell clones maintain their membrane phenotype and pattern of cytokine secretion and express a TCR identical to the TCR of the parental nontransformed clones. Most importantly, the TCR of the transformed clones is functional in signal transmission as demonstrated by antigen-induced MHC-restricted proliferation and production of the cytokines IFN-γ and lymphotoxin.

Human T-cell clones have been immortalized with human T-cell leukemia virus I (HTLV-I) (20–23). One disadvantage of HTLV-I transformation is that the infected T cells may lose their cytotoxic potential and CD3–TCR complex after 2–4 months (22, 23). Furthermore, HTLV-I-transformed T cells produce infectious retroviruses (24–26).

T-cell transformation by HVS is not burdened by these problems. We have cultured transformed clones for >1 year without losing TCR expression or transduction. Moreover, HVS-transformed T-cell clones are unable to produce infectious virus, as indicated by serial coculture with permissive owl monkey kidney cells. Thus far, none of our HVS-transformed long-term T-cell lines, which are regularly tested for HVS production, has shed detectable amounts of infectious virus, not even after antigen-specific stimulation of the T cells. We recommend, however, that HVS-transformed T-cell lines be handled as potentially infectious material. Special caution and containment facilities are recommended for the infection and initial propagation of the T cells.

HVS is a simian lymphotropic γ-2 herpesvirus (27) that is able to transform T cells from nonhuman primates (28, 29). In many New World monkey species, HVS causes fulminant lymphomas and leukemias. The oncogenic potential has been mapped to the left terminus of the genome (29, 30). One left-terminal open reading frame coding for the saimiri transformation-associated protein STP is able to cause morphological transformation of rodent fibroblasts (31). Virus strain 488 of HVS subgroup C also transforms human PBMCs to continuous growth (4).

By using HVS-transformed human T-cell clones of undetermined antigen specificity, it has been shown that (*i*) T cells can be immortalized directly from peripheral blood cells and thymocytes without prior antigen-specific selection (4), (*ii*)

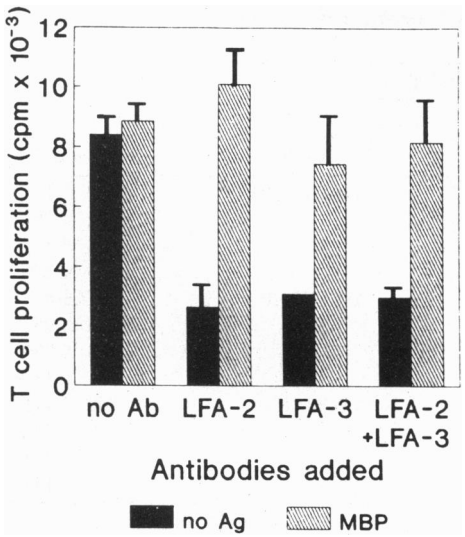

FIG. 2. Antigen-induced proliferation of the HVS-transformed T-cell clone SSBP8T. Background proliferation (solid bars) was reduced in the presence of mAbs against LFA-2 (CD2), LFA-3 (CD58), or both (final concentration, 2 μg/ml). MBP (30 μg/ml) was presented on HLA-DRA/DRB5*0101-transfected L cells. Proliferation was determined by [³H]thymidine incorporation. Ab, antibody; Ag, antigen.

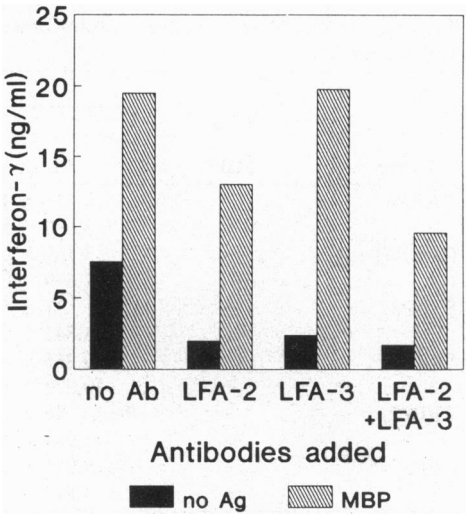

FIG. 3. Antigen-induced production of IFN-γ by the HVS-transformed T-cell clone SSBP8T. Background secretion (solid bars) was reduced in the presence of mAbs against LFA-2 (CD2), LFA-3 (CD58), or both (final concentration, 2 μg/ml). MBP (30 μg/ml) was presented on HLA-DRA/DRB5*0101-transfected L cells. Supernatants were collected after 48 h and serial dilutions were measured in duplicate by ELISA. The standard deviation of the day-to-day control was 8%. Ab, antibody; Ag, antigen.

both CD4+ and CD8+ T cells can be immortalized with HVS (4), and (*iii*) HVS-transformed T cells grow via an autocrine mechanism, in response to activation signals mediated by their CD2 molecules during mutual cell–cell contact (32). Consistent with this mechanism, our antigen-specific HVS-transformed T-cell clones showed a high spontaneous proliferation and cytokine secretion tending to obscure antigen specificity. Background activity was particularly pronounced when human PBMCs were used as APCs. In contrast, when DR-transfected mouse L cells were used as APCs, the background proliferation and cytokine production were lower. This background could be further reduced in the presence of mAbs against CD2, LFA-3 (CD58), or both. The T-cell clones studied were specific for MBP, a well-characterized myelin protein that induces one of the best-characterized experimental autoimmune diseases, experimental autoimmune encephalomyelitis (7, 33, 34). Further, MBP is a prominent candidate autoantigen for multiple sclerosis, the most important human demyelinating disease of presumed autoimmune pathogenesis (35). Consequently, the human T-cell response against MBP and its physiological and pathological regulation are a major focus of current multiple sclerosis research (5, 6, 10). We anticipate that HVS-transformed human T-cell clones specific for MBP or other human autoantigen will become a very useful tool for many applications. For example, transformation with HVS should permit production of essentially unlimited amounts of autoantigen-specific TCRs, which could be used for studies of antigen-specific immunoregulation *in vitro* (36) and, eventually, for TCR-targeted immunotherapy *in vivo* (33, 34). Further, HVS transformation should allow the production of the large numbers of functional autoantigen-specific human T cells that are required for transfer of autoreactive human T-cell clones into experimental animals, such as severe combined immunodeficiency mouse–human chimeras, HLA-DR-transgenic mice, or MHC class II compatible primates.

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