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Contents Volume 174 · 1987

Short Communications

Letter to the Editor

Authors' Index

Subject Index

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Stable Expression of Clonal Specificity in Murine Cytomegalovirus-Specific Large Granular Lymphoblast Lines Propagated Long-Term in Recombinant Interleukin 2

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Abstract

The somatic stability of cloned long-term cytolytic T lymphocyte lines (CTLL) specific for antigens encoded by murine cytomegalovirus (MCMV) was tracked for more than two years of continuous *in vitro* propagation. Clone S1 retained its original specificity for a structural (S) antigen of MCMV for about eight months in the presence of antigen and interleukin 2 (IL 2), but not in IL 2 alone. In the following months, however, in spite of the continued presence of antigen, clonal variants developed that displayed distinct patterns of target cell recognition, including loss of the original specificity and acquisition of exclusive specificity for the natural killer target cell YAC-1. On the other hand, large granular lymphoblast (LGL) lines, line IE1-IL and a series of sublines thereof, could be established that stably expressed L^d-restricted specificity for a viral nonstructural immediate-early (IE) antigen more than two years after withdrawal of antigen and feeder cells when propagated in the presence of pure recombinant human IL 2. The finding that the presence of antigen was not essential for the stability of clone IE1-derived CTLL indicates that maintenance of specificity in LGL lines is not a result of antigen-mediated selection, but reflects an intrinsic property.

Introduction

Based on different *in vitro* propagation requirements, three types of CTLL have been distinguished (for review, see ref. 1). Type 1 CTLL do not require external supply of IL 2, but depend on the presence of antigen. Such a state lasts only for a few weeks. Type 2 CTLL depend on both, antigen and IL 2, and can then be maintained for several months. Most long-term virus-specific CTLL reported so far belong to this group (2–5). Finally, CTLL growing in lymphokine-supplemented medium in the absence of feeder cells and antigen (6–8) are referred to as type 3 CTLL. Since virus-

Abbreviations: Con A = concanavalin A; *CTL* = cytolytic T lymphocyte(s); *CTLL* = CTL line(s); *IE* = immediate-early; *IFN* = interferon; *IL 2* = interleukin 2; *LGL* = large granular lymphoblast(s); *LPS* = lipopolysaccharide; *lsIL 2* = lymphokine supernatant IL 2; *MCMV* = murine cytomegalovirus; *MHC* = major histocompatibility complex; *NK* = natural killer; *PFU* = plaque-forming units; *rhIL 2* = recombinant (DNA) human IL 2; *U* = units.

specific type 3 CTLL can be propagated in any laboratory without the need to establish the technology for viral infection, it should be an aim to select such CTLL as reference lines for the definition of virus-encoded epitopes.

When compared with type 2 CTLL, however, the selection of influenza virus-specific type 3 CTLL proved to be a rare event (9), and type 3 clones in general were found to display profound changes in the karyotype (10), frequently associated with expression of nonspecific cytolytic activity (for review, see ref. 11). Degeneration of specificity to an NK-like (12), an aged killer (13) or an indiscriminate, promiscuous target cell recognition pattern was found to be accompanied by the acquisition of LGL morphology and an NK cell surface marker profile (for review, see ref. 14). It has been proposed recently that during adaptation to antigen-independent long-term growth in the only presence of lymphokines, CTLL invariably and rapidly lose the original specificity, supposedly due to overgrowing specificity loss variants (15).

In this communication, we report on virus-specific type 3 CTLL with LGL morphology that could be established with relative ease and did not acquire the promiscuous reactivity predicted. Clone IE1, specific for the viral nonstructural IE membrane antigen of MCMV (16, 17), was selected in July 1984 (18), and deprived of antigen and feeder cells in November 1984. Since then, line IE1-IL and sublines thereof have been maintained continuously in rhIL 2 till now (March 1987) without losing viral antigen specificity. The existence of such an LGL line contradicts published opinions. First, an LGL phenotype is not necessarily associated with nonspecific cytolytic activity, and second, antigen is not required for the maintenance of specificity in CTLL.

Materials and Methods

Propagation of CTLL

Technical details regarding the selection of MCMV-specific CTL clones were given in a previous report (18). Line S1-A was split twofold in 6-day intervals and restimulated in 2-ml culture with 1×10^6 30-Gy γ-ray-irradiated splenocytes, derived from MCMV-infected BALB/c (MHC-d) mice, and 1×10^5 PFU of infectious MCMV (ATCC VR-194, Smith strain) in the presence of 50 U of rhIL 2 per ml. Line IE1-IL and its sublines were split likewise and maintained in the presence of 50 U of rhIL 2 per ml without restimulation. The number of restimulations as well as of passages with IL 2 alone is coded in the form An_1ILn_2 with full stops indicating recloning (e.g. code A22IL11.9 signifies that the line has been recloned after 22 restimulations followed by 11 passages in IL 2 alone, and was then maintained in IL 2 without antigen for further 9 passages). Lot 89050/84802 of rhIL 2 (Sandoz Forschungsinstitut, Vienna, Austria) is > 99 % pure IL 2 (1.04 ± 0.35 ng of LPS per mg of protein) with a specific activity of 7.3×10^6 U/mg of protein normalized to a reference standard (lot ISP-841) supplied by the Biological Resources Branch at the National Cancer Institute, Frederick, MD, U.S.A.

Recloning and statistical analyses

Recloning was routinely done by seeding 0.5 cells per round-bottomed 96-tray (0.2 ml) microtiter well. The clone probability was calculated from the Poisson equation (19). In split

assays (see Table 1), the probability for an independent distribution of distinct monospecific clones (null hypothesis) was obtained by applying Fisher's exact probability test (20).

Target cells and cytolytic assay

The Moloney leukemia virus-induced T cell lymphoma of A/Sn (MHC-a, K^kD^d) mice, YAC-1, was used to probe NK activity, and the methylcholanthrene-induced mastocytoma of DBA/2 (MHC-d) mice, P815, served to detect aged killer reactivity. Simian virus 40 (SV40)-transformed kidney cells of B10.D2 (MHC-d) origin, KD2SV (21), and bovine papilloma virus-transformed fibroblasts derived from DBA/2 mice, DBA/2-BPV (provided by Dr. H. Pfister, Erlangen, F.R.G.), were used as probes for MCMV-specific CTL after either MCMV infection (multiplicity of 20 PFU/cell) under conditions of selective and enhanced expression of IE genes (IE-KD2SV and IE-DBA/2-BPV) or presentation of MCMV structural proteins (S-KD2SV) (16, 17). Specific lysis was determined in a standard 3-h ^{51}Cr release assay.

Cell surface phenotyping

For cytofluorometric analysis of asialo-GM1 expression, the CTL were labeled with rabbit anti-asialo-GM1 serum gammaglobulin (0.1 mg per 10^6 cells; code no. 014-09801, Wako Pure Chemical Industries, Osaka, Japan). Fluorescein-conjugated $F(ab)_2$ fragments of goat anti-rabbit IgG (H and L chain-specific; Cat. No. 1312-0081, Cappel, Malvern, PA, U.S.A.) was used as second antibody. The relative fluorescence intensities of 10^4 viable cells measured with a fluorescence-activated cell sorter (FACS IV; Becton Dickinson FACS Systems, Sunnyvale, CA, U.S.A.) were expressed in a linear scale of 255 channels, and the fluorescence in the channel that separated the population in two halves is given in Table 5 to represent the median fluorescence of the population.

Assay for IFN

The supernatants of ten IE1-IL CTL cultures were pooled 4–6 days (about one population doubling) after seeding of 10^5 cells. At the time of harvesting, the differences in the average cell numbers between different lines did not exceed 40%. The IFN assay was performed essentially as described (22). IFN titers were expressed in international reference units (IU/ml) of murine IFN (NIH standard G-002-904-511). Sensitivity to pH 2 characterized the IFN as IFN-γ. This result was confirmed by complete neutralization of the IFN activity with monoclonal anti murine IFN-γ antibody (hybridoma R4-6A2 secreting IgG1; kindly provided by Dr. Havell, Saranac Lake, NY, U.S.A.).

Results

Selection and attributes of the CTL clones S1 and IE1

The viral antigen specificity of these two clones has been reported previously (18). Clones were derived from mesenteric lymph nodes of 10-month-old BALB/c mice which had been infected with MCMV as newborns and had established a latent infection after a period of persistent viral replication. Already when clones became first visible in microcultures after only five *in vitro* restimulations could two discrete morphologic phenotypes be distinguished: clones of large lymphoblasts and clones of small, densely growing lymphocytes. After six months of periodic restimulation (stage A30), the cloned lines IE1-A (Fig. 1a) and S1-A (Fig. 1b) were inspected by electron microscopy. IE1 cells showed the characteristics of LGL with typical cytolytic granules (23), while S1 cells were considerably

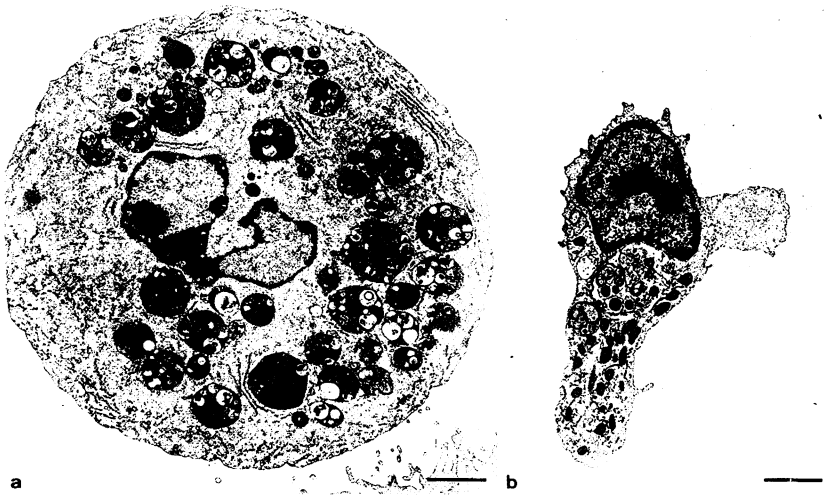

Fig. 1. Morphologic phenotype of the MCMV-specific CTL clones IE1 and S1. Electron microscopic analysis of clone morphology. Clone IE1 cells (a) are large granular lymphoblasts with a diameter of 16–17 μm, whereas clone S1 cells (b) are considerably smaller in size and display a pronounced protopode-uropode polarity. Both bars represent 2 μm. Ultrathin sections were stained with uranyl acetate and lead citrate.

smaller and displayed a pronounced polarity with a protopode occupied by the nucleus, a uropode containing the organelles, and one or more pseudopodia. The morphologic characteristics remained stable in clone S1 for about eight months and apparently indefinitely in the LGL clone IE1. Both clones recognized a viral antigenic determinant specified by MCMV, the epitope S1 of a structural antigen of the virus and the nonstructural IE epitope IE1, respectively, in conjunction with cellular (D)L^d class I glycoprotein, and expressed the surface markers Thy-1.2 and Lyt-2.2, but not Lyt-1.2 and L3T4 (18). Clone IE1 was recently found to recognize L fibroblasts (MHC-k) cotransfected with the L^d gene and the major IE gene of MCMV, gene ie1, that codes for the regulatory, intranuclear phosphoprotein pp89 (24). Neither of these clones lysed Con A-induced lymphoblasts of the congenic mouse strains B10, B10.D2, B10.S, B10.BR, B10.Q, B10.M, B10.P, and B10.RIII (b, d, s, k, q, f, p, and r MHC haplotypes, respectively).

Morphologic alteration and somatic variation of specificity within line S1-A

Line S1-A proved to be dependent on both, IL 2 and antigen. In the presence of IL 2 alone, cytolytic activity was gradually lost within two months (not shown). Continuous selection by restimulation with infectious virus and irradiated, primed splenocytes resulted in retention of cytolytic

activity, but not specificity. After 15 months of propagation (stage A78), the line displayed morphologic heterogeneity with a high proportion of LGL, and had acquired reactivity against the lymphoma YAC-1. The synchronism of these alterations along with published experience concerning the reactivity of LGL clones (reviewed in ref. 14) suggested LGL to be the mediators of that NK-like activity. To test this prediction, LGL clones and uropodial lymphocyte clones were separated by recloning and probed for cytolytic activity against the specific target S-KD2SV and the prototype NK target YAC-1 (Table 1). It was found that the parental line at that stage comprised four types of clones: the original type consisting of S-specific uropodial lymphocytes and, in addition, uropodial lymphocytes as well as LGL with dual specificity and, against all prospects, S-specific LGL. This result demonstrated that either reactivity can be associated with either morphologic phenotype, and thus proved that both alterations are independent events.

With the aim to rescue clone S1 in its original form, long-term lines were established from the four S-specific, uropodial subclones (see Table 1) of line S1-A (U1–U4, Table 2). When the lines were tested 3.5 months after recloning (stage A78.18), line S1-A-U4 had already begun to switch to the LGL phenotype, while line S1-A-U3 retained the original morphology for at least three further months. The reactivity pattern observed at stage A78.34 typified line S1-A-U1 as NK-like with dual specificity for the

Table 1. Independent segregation of morphologic phenotype and NK-like reactivity within line S1-A

Morphologic phenotype	Number of clones	Proportion of negative cultures (clone probability)	Median value (range) of % specific target cell lysis	
			S-KD2SV	YAC-1
Large granular lymphoblast	24 [2][a]	348/384 (0.952)	29 (13–53)	20 (10–40)
	12		26 (17–35)	< SL[b]
Uropodial lymphocyte	9 [0][a]	371/384 (0.983)	59 (46–65)	38 (11–52)
	4		36 (21–60)	< SL[b]

Note: At stage A78, line S1-A was recloned by seeding 0.5 cells in a 0.2 ml microculture along with feeder cells and infectious MCMV as antigen. After 3 weeks, microcultures screened positive for growth were split and quarter aliquots tested for cytolytic activity.

[a] Data in brackets represent the statistically expected number of double-positive cultures as a consequence of non-clonality. Lysis of S-KD2SV and YAC-1 was mediated by the same clone, because the observed number of double-positive cultures could not be explained statistically ($P < 10^{-9}$, Fisher's test).

[b] Significance limit (upper 99 % tolerance limit of spontaneous lysis) that was 5.2 %.

Table 2. Somatic variation of specificity in S1-A sublines

S1-A subline	Morphologic phenotype[a]	Protocol code	Target cells (% specific lysis at E/T ratios of 10–2.5–0.6:1)[b]			
			S-KD2SV	KD2SV	YAC-1	P815
S1-A-U1	UL	A 78.18	32–17–9	< SL[c]	11–5–3	–[d]
	UL	A 78.26	25–15–8	< SL	11–8–4	< SL
	UL-LGL	A 78.34	29–19–16	< SL	22–13–3	< SL
S1-A-U2	UL	A 78.18	34–13–7	< SL	6–4–1	–
	UL	A 78.26	27–21–11	8–5–0	13–9–4	< SL
	UL-LGL	A 78.34	35–33–25	23–10–4	23–21–15	< SL
S1-A-U3	UL	A 78.18	26–17–7	< SL	34–18–6	–
	UL	A 78.26	27–15–11	< SL	22–12–8	< SL
	UL	A 78.34	40–32–18	17–13–6	44–33–25	< SL
S1-A-U4	UL-LGL	A 78.18	40–18–12	< SL	21–11–5	–
	LGL	A 78.26	< SL	< SL	20–10–3	< SL
	LGL	A 78.34	< SL	< SL	31–20–12	< SL

[a] Uropodial lymphocyte (UL) or large granular lymphoblast (LGL), or intermediate forms (UL-LGL).
[b] Mean value of six replicate determinations for each effector-to-target-cell (E/T) ratio tested.
[c] Specific lysis below the significance limit (upper 95 % confidence limit for the mean value of spontaneous lysis) at the highest E/T ratio tested.
[d] Not determined.

specific target and YAC-1. Lines S1-A-U2 and U3 represent a novel type of CTL characterized by the potential to lyse non-infected fibroblasts (including non-transformed embryonal fibroblasts, not shown) which are resistant to YAC-type NK reactivity. The genesis of line S1-A-U4 proved that NK reactivity can be mediated by cells of the CTL lineage, as the original specificity for S antigen of MCMV converted into an exclusive specificity for YAC-1. None of those variants lysed the NK-resistant aged killer target cell P815 (13).

In conclusion, attempts to recover the original form of clone S1 failed and, instead, three distinct target cell recognition patterns were found to have developed in formerly antigen-specific S1-A subclones.

Stability of specific cytolytic activity in long-term IE1 CTLL maintained in rhIL 2

As with clone S1, clone IE1 was first propagated by repeated restimulations. At stage A22, line IE1-A was split, and a subline, designated IE1-IL, was maintained without virus or feeder cells in the presence of rhIL 2. After withdrawal of IL 2, IE1-A and IE1-IL cells inevitably died within 48 h, demonstrating that both lines were IL 2-dependent. When assayed for

Table 3. Long-term stability of lytic activity and specificity within IE1 lines maintained in rhIL 2

Experiment	Effector cell line	Protocol code	Target cells (% specific lysis at E/T ratios of 10–2.5–0.6:1)[a]				
			IE-KD2SV	S-KD2SV	KD2SV	YAC-1	P815
1	IE1-A	A42	35–15–3	2	0	2	0
	IE1-IL	A22IL.20	39–17–6	3	1	0	1
	IE1.14-IL	A22IL11.9	57–33–11	4	2	2	0
	IE1.18-IL	A22IL11.9	43–25–8	1	1	2	3
	IE1.21-IL	A22IL11.9	56–35–14	4	2	4	2
2	IE1.14-IL	A22IL11.39	49–29–12	3	1	2	2
	IE1.18-IL	A22IL11.39	41–24–11	2	0	0	1
	IE1.21-IL	A22IL11.39	36–30–22	0	2	1	0
	IE1.18.15-IL	A22IL11.21.18	63–46–35	0	1	2	0
	IE1.18.20-IL	A22IL11.21.18	70–61–32	3	3	1	2
	IE1.18.34-IL	A22IL11.21.18	51–44–31	1	4	2	2

[a] Mean values of six replicate determinations. In case of non-significant lysis, only the value for an effector-to-target-cell (E/T) ratio of 10 is shown.

specificity after 20 passages in rhIL 2 (4 mo, stage A22IL20), line IE1-IL displayed the same specificity pattern as did line IE1-A that had been further restimulated (Table 3, Expt. 1). In detail, both lines lysed KD2SV fibroblasts only when MCMV IE genes were expressed (target IE-KD2SV, but not KD2SV and S-KD2SV), and not YAC-1 or P815. After 11 passages in rhIL 2, line IE1-IL was recloned (26 of 384 cultures positive for growth; clone probability 0.965). Non-lytic variants were not observed, and all subclones had retained the original specificity. Three representative sublines (IE1.14-IL, IE1.18-IL, and IE1.21-IL) were established by long-term propagation in rhIL 2, and assayed together with the parental lines at stage A22IL11.9 (Table 3, Expt. 1) as well as six months later at stage A22IL11.39 (Table 3, Expt. 2). At stage A22IL11.21, subline IE1.18-IL was again recloned (37 of 384 cultures positive for growth; clone probability 0.950), and sublines thereof (IE1.18.15-IL, IE1.18.20-IL, and IE1.18.34-IL) were assayed at stage A22IL11.21.18 after a total of 50 passages (10 months) in rhIL 2 without antigen (Table 3, Expt. 2). All sublines displayed exactly the specificity pattern of the parental lines, and none had developed NK-like, aged-killer, or promiscuous reactivity. This demonstrated that IL 2 was necessary and sufficient for the maintenance of cytolytic activity and specificity in the LGL clone IE1 and all its descendants.

Retention of specificity in presence of high doses of rhIL 2

Recent findings have indicated the induction of NK-like and promiscuous reactivity in antigen-specific CTL clones by high doses of lymphokines

(12, 14). We therefore tested the disposition of the established CTLL IE1.18-IL to alter the pattern of target cell recognition under the influence of high doses of rhIL 2. Table 4 documents that the specificity remained stable when the line was grown for one month (6 passages) in 500 U of rhIL 2 per ml (4.4 nM), tenfold the amount used routinely for propagation. After two weeks, part of the line was exposed for further two weeks to 5000 U of rhIL 2 per ml, again without induction of significant NK-like or promiscuous reactivity. In detail, KD2SV as well as DBA/2-BPV fibroblasts were lysed only after expression of MCMV IE genes, and YAC-1 or P815 were not lysed. The same specificity pattern was observed when rhIL 2 was replaced for one month by 40 % supernatant of lectin (Con A)-induced rat lymphoblasts (lsIL 2) known to contain a variety of lymphokines. These data imply that line IE1.18-IL was refractory to an IL 2-mediated change in specificity. It should be noted that prolonged propagation in lsIL 2 (50 U of IL 2/ml) resulted in a reduced growth and ultimately in the loss of the subline, suggesting that the use of rhIL 2 was essential for the long-term maintenance of line IE1-IL.

Somatic variation of asialo-GM1 expression and IFN-γ secretion in line IE1-IL

The glycolipid asialo-GM1 has been regarded as a surface marker for NK-cells (25). Previous studies have shown that long-term CTLL can also display this marker (14). The demonstration of constitutive asialo-GM1 expression in highly specific CTLL disproves a causal linkage between the presence of this glycolipid and NK-like function (compare Tables 4 and 5). At stage A22IL64, line IE1.18-IL and all its sublines still expressed Thy-1.2 and Lyt-2.2 antigens (not shown), but varied in the degree of asialo-GM1

Table 4. Invariant antigen specificity of line IE1.18-IL during exposure to high doses of IL 2

Protocol code for line IE1.18-IL	Lymphokine (U of IL 2/ml)	Target cells (% specific lysis at E/T ratios of 10–1–0.1:1)[a]					
		IE-KD2SV	KD2SV	IE-DBA/2-BPV	DBA/2-BPV	YAC-1	P815
A22IL11.53	rhIL 2 (50)[b]	50–45–20	2	52–35–16	0	1	1
A22IL11.47 (6)[c]	rhIL 2 (500)[b]	46–34–14	1	48–31–11	3	6	2
A22IL11.47 (3/3)	rhIL 2 (500/5000)	31–10–5	3	47–17–1	0	4	3
A22IL11.47 (6)	lsIL 2 (200)[d]	37–23–11	2	45–25–2	1	3	2

[a] Mean values of six replicate determinations.
[b] 6.8 and 68 ng (0.44 and 4.4 pmol) of pure IL 2/ml, respectively.
[c] Numbers in parentheses indicate the number of passages in the high dose of lymphokine noted in the second column.
[d] Lymphokine supernatant of Con A-stimulated rat lymphoblasts (lsIL 2), containing 500 U of IL 2/ml and 54 IU of IFN-γ/ml besides other lymphokines that have not been quantitated. Residual Con A activity was blocked by adding α-methyl-mannoside (50 mM).

Table 5. Expression of the NK-cell surface marker asialo-GM1 and secretion of IFN-γ by IE1-IL sublines

IE1-IL subline (U of rhIL 2/ml)	FACS median channel fluorescence[a]		Titer of IFN-γ[b] IU/ml
	Control	anti asialo-GM1	
1.18-IL (50)	20	91	54; 36 (<3; <3)
1.18.15-IL (50)	25	163	54; 54 (<3; <3)
1.18.20-IL (50)	15	26	18; 36 (<3; <3)
1.18.34-IL (50)	30	38	12; 12 (<3; <3)
1.18-IL (500)	22	231	162; 162 (<3; <3)[c]

[a] Results are expressed as relative fluorescence intensities on a linear scale with 10^4 cells analyzed. Staining with FITC-labeled second antibody alone served as control.

[b] Double determination. Values in parentheses represent the IFN titer after pH2 treatment. The detection limit was 3 IU/ml. Medium and rhIL 2 did not contain IFN.

[c] Control experiments with up to 1 μg of LPS per mg of IL 2 ensured that LPS present in the rhIL 2 preparation (1 ng per mg of IL 2) did not induce IFN.

expression. The fluorescence profile of line IE1.18-IL (not depicted) indicated that the population comprised individual cells with an asialo-GM1 expression ranging from almost negative to very high. On the population level, sublines stably displayed a higher (IE1.18.15) or lower (IE1.18.20 and IE1.18.34) median fluorescence intensity than did the parental line. Expression of the glycolipid was elevated after exposure of the line for one month to 4.4 nM rhIL 2 (500 U/ml).

The variation in the amount of asialo-GM1 detected at the cell membrane corresponded with the secretion of IFN-γ. When maintained in 0.44 nM rhIL 2, all lines displayed a low basic level of IFN-γ secretion that was enhanced by cultivation in higher doses of IL 2.

These findings indicated that somatic variation did occur in line IE1-IL, but, apparently, antigen recognition specificity was conserved.

Discussion

The selection of antigen-independent antiviral CTLL with constant specificity appears to be a rare event. TAYLOR and ASKONAS have pointed out in a recent review that they could keep only a single influenza-specific antigen-independent CTLL sufficiently long enough for detailed analysis, though a series of antigen-dependent clones were obtained with ease (9). Our long-term study on the cloning of CTL specific for antigens specified by MCMV, a member of the herpes virus group, has now documented long-term stability of antigen-independent CTLL in an essentially different virus system, thus confirming that selection of stable virus-specific type III CTLL is in principle reproducible. To our knowledge, the existence of antigen-independent CTLL has not been reported before for any other

herpes virus. With respect to the continuity of MHC-restricted cytolytic specificity in the absence of antigen and feeder cells, line IE1-IL and descendants thereof are without precedent among virus-specific CTLL. It is an important aspect that previous attempts to maintain CTLL have employed lsIL 2 comprising a variety of lymphokines/monokines, while line IE1-IL was propagated in purified rhIL 2. We have experienced that replacement of rhIL 2 by lsIL 2 did not induce rapid alterations in the specificity or cytolytic activity of line IE1.18-IL (Table 4), but caused reduced growth, and ultimately resulted in the extinction of the line. The use of rhIL 2 may therefore be crucial for a successful maintenance of antigen-independent CTLL.

It has been proposed recently that stable expression of specificity in long-term CTLL is a matter of antigen-mediated selection against clonal specificity loss variants rather than the consequence of structural stability of the idiotypic T cell receptor (15). Consistent with that idea, line S1-A retained specificity only when restimulated periodically with viral antigen. Later on, however, and without any apparent change in the propagation conditions, a spectrum of variants developed, including LGL that had lost the original specificity and had instead attained specificity for the NK-target YAC-1. Thus, somatic variation in line S1-A was finally able to override the counterselection by antigen. This proved to be a slow process, since recloning revealed a coexistence of ancestor-type CTL and variant CTL seven months after the first signs for specificity drift in that line.

It is evident from our data that selection by antigen can be excluded as an explanation for the stability of specificity displayed by line IE1-IL. The objection that this clone might be autostimulatory by constitutively expressing MCMV IE antigen can be refuted, because neither viral nucleic acid nor IE protein (pp89) was detectable (not shown). Transformation does not offer an alternative explanation either, since after withdrawal of IL 2 all IE1-IL sublines inevitably died. Such a strict IL 2-dependence is a strong criterion for a nontransformed state.

It appears that uropodial type II CTLL have a programmed tendency to switch to the LGL phenotype that is characterized by a higher degree of cytoplasmic organization (Fig. 1), while a revert to uropodial morphology was not observed. This unidirectional development suggests that LGL represent the more mature, more specialized, and perhaps also the more stable type of CTL. The morphologic difference between antigen-dependent type II and antigen-independent type III CTLL has been recognized before (7, 9). In this context, it is important to point out that clone IE1 was isolated from lymph nodes of latently MCMV-infected mice already as an LGL. Latent herpes virus infection provides a unique condition for the *in vivo* development of CTL clones, as reactivation events can result in frequent *in vivo* restimulation, generating CTLL *in vivo*. It could be speculated that selective expression of IE antigens during reactivation from viral latency (26) selectively favors the *in vivo* maturation of IE-specific

CTL clones, while S-specific CTL clones must be expected to outlast in a less developed state. Altogether, the genesis of clone IE1 suggests that stable type III CTL clones exist *in vivo* and thus do not represent an artificial state attained only *in vitro* during long-term propagation.

We hypothesize that, *in vivo* and *in vitro,* CTL clones undergo frequent somatic variation until reaching a more stable functional phenotype. This state appears to be signified by LGL morphology, regardless of the final recognition specificity. Cloning of those LGL whose terminal functional phenotype is the expression of MHC-restricted antigen-specificity should result in stable, IL 2-dependent CTLL which can then serve as certifiable probes for the definition and identification of antigens. Work to test this prediction is in progress.

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