CORRELATED EXPRESSION OF T CELL GROWTH FACTOR 
DEPENDENCE, SENSITIVITY TO VICIA VILLOSA 
LECTIN, AND CYTOLYTIC ACTIVITY IN HYBRIDS 
BETWEEN CYTOLYTIC T CELLS AND T LYMPHOMAS*

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The use of conditioned media from leukocyte cultures activated by T cell mitogens 
(1, 2) has allowed the derivation of cloned cell lines from cytolytic T lymphocytes 
(CTL),¹ which stably express antigen-specific cytolytic activity (3–5). The growth-
promoting activity of conditioned media for CTL lines is called T cell growth factor 
(TCGF) or interleukin 2. It has been purified and consists of a glycoprotein (gp) with 
a weight of 15 kilodaltons (kd) in man and rat (6, 7) and of 30 kd in the mouse (8). 
The action of TCGF is mediated through specific surface receptors that become 
expressed on T lymphocytes upon exposure to antigenic stimulator cells or mitogens 
(9–11). In the experiments to be described in this paper we have used the crude 
supernatant of concanavalin A (Con A) -stimulated rat spleen cells (CS) as a source 
of TCGF. Cloned CTL lines can be derived from CTL-containing mixed lymphocyte 
cultures either by immediate cloning in the presence of TCGF and feeder cells (CTL-
A) or by maintaining cell populations in TCGF for several months before cloning in 
TCGF without feeder cells (CTL-B) (for discussion see ref. 12). CTL-B lines differ 
from normal CTL and CTL-A lines in morphology and karyotype (13) and by the 
fact that their growth is not dependent on feeder cells or on any growth factor besides 
TCGF. When CTL lines are plated in the absence of TCGF, cell growth stops 
reversibly in G1 (14; R. Sekaly, unpublished observations).

We have reported previously that somatic cell fusion of TCGF-dependent murine 
CTL-B or CTL-A lines with TCGF-independent murine T lymphomas yields two 
types of hybrids (5, 12). When the culture medium used during the hybrid selection 
contains CS, the majority of the isolated hybrids is cytolytic and dependent on CS 
(CS⁺). Most of these hybrids stably maintain their phenotype. On the other hand, 
selection in medium without CS only yields noncytolytic, CS-independent (CS⁻)

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¹ Abbreviations used in this paper: Con A, concanavalin A; CS, supernatant from Con A-stimulated rat 
spleen cells; CS⁺, CS independent; CS⁻, CS dependent; CTL, cytolytic T lymphocyte; gp, glycoprotein; 
HAT, hypoxanthine, aminopterin, thymidine; kd, kilodaltons; PBS, phosphate-buffered saline; TCGF, T 
cell growth factor; VV, Vicia villosa lectin.

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hybrids. These hybrids also maintain their phenotype in that they do not become cytolytic when grown in presence of CS. Thus, these characteristics are fixed and inherited over many cell generations. We decided to test the hypothesis that one type of hybrid is derived by chromosomal segregation from the other type. Under this assumption, it appeared more probable that CS− hybrids are derived from CS+ than vice versa, because (a) we consistently found significantly more hybrids when selecting in CS than without CS, regardless of whether CTL lines were fused with a diploid or tetraploid T lymphoma line, and (b) we did not obtain CS− hybrids in a cross involving a tetraploid CTL line and a diploid T lymphoma line (12). Therefore, we hypothesized that CS+ hybrids contain a CTL line chromosome carrying one or several genes that render a cell CS+ and that both copies of this chromosome are lost in the CS− hybrids. Here we report on the isolation and characterization of CS− variants derived from cloned CS+ hybrids.

The striking correlation between the expression of CS dependence and cytolytic activity induced us to examine whether other markers typical of CTL lines continue to be expressed in the cytolytic hybrids and are lost in CS− variants. T130 and T145 have been described as surface gp specific for resting and mature cytolytically active T lymphocytes in the mouse, respectively (15, 16). We have previously reported (17) that murine CTL lines express T145 but not T130, whereas murine T lymphomas contain T130 but not T145. It is possible that T145 and T130 represent differently glycosylated forms of the same protein, but it is not clear whether the expression of T145 is important for the cytolytic function (17). The search for a specific reagent for T145 led to the discovery of a lectin from *Vicia villosa* (VV), which has high affinity for terminal N-acetyl-D-galactosamine (18) and binds T145 but not T130 of the lymphoblasts contained in mixed leukocyte cultures (19). All CTL lines tested so far bind high amounts of VV and are very susceptible to its cytotoxic effect, whereas T lymphomas bind 500–1,000-fold less VV and are highly resistant (17). Therefore, we have compared the sensitivity to VV of CS+ hybrids and CS− variants.

**Materials and Methods**

**Cell Culture and Cell Lines.** The culture medium used for all cell types was Dulbecco's modified Eagle's medium supplemented as previously described (20), containing 5% fetal calf serum. CS was produced by culturing rat spleen cells from outbred OFA rats in this medium with 5 μg/ml of Con A for 48 h. For the growth of TCGF-dependent cell lines, a final concentration of 25% of this crude supernatant was added to the culture medium. All cell lines and hybrids were grown in 60-mm plastic tissue culture dishes and subcultured twice weekly by splitting at ratios of 1:10–1:50. Hybrids, clones, and hybrid variants isolated in 96-well microtiter plates in the presence of irradiated macrophages were grown in 24-well plates (Costar, Data Packaging, Cambridge, MA) for 1 wk before being transferred into petri dishes. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO2.

B6.1.SF.1 is a cloned murine CTL line derived from a female C57BL/6 mouse (21). At the time of fusion it had been in culture for >2 yr and had strong cytolytic activity against the BALB/c myeloma S194. BW5147 is a spontaneous T lymphoma from an AKR mouse. A thioguanine- and ouabain-resistant double mutant from the BW5147 T cell lymphoma was obtained from B. Hyman, The Salk Institute, San Diego, CA. B6.1.SF.1 and the TCGF-dependent hybrids are slightly adherent and were removed by a 5-min incubation in EDTA (0.2 g/l) in phosphate-buffered saline (PBS). Cells were checked monthly for mycoplasma contamination by the method of Fogh (22) as modified by J. W. Stocker (personal communication) and were always found to be mycoplasma free.

**Production of Hybrids.** Before fusion, the BW5147 line was grown for two passages in medium
containing thioguanine and ouabain to eliminate any revertants. Exponentially growing B6.1.SF.1 (8 × 10^6) and BW5147 (10^7) cells were fused using 45% polyethylene glycol in a procedure essentially identical to the one described by Galfré et al. (23). Hybrids were selected in culture medium containing 5 × 10^-4 M hypoxanthine, 5 × 10^-7 M aminopterin, 1.6 × 10^-6 M thymidine (HAT) and 10^-3 ouabain containing 25% of CS. Aliquots of 2 × 10^6 cells (based on the initial concentration of the fusion mixture) were dispensed into flat-bottomed wells of 96-well microtiter plates (Costar, Data Packaging) containing 10^4 irradiated (2,000 rad) C57BL/6 mouse peritoneal macrophages (3), in a total volume of 200 μl of selective medium. Cells were fed weekly by replacing ~½ of the selective medium. After 25 d, growing hybrids from 10 different wells were detached by vigorous pipetting with a micropipette and half of the cells were used to measure the cytolytic activity of the hybrids (see below).

Assay of Cytolytic Activity. Culture medium was used throughout the test. Cells were harvested, washed twice, counted, and placed into round-bottomed 96-well microtiter plates. After serial dilution of the cells, 10^4 51Cr-labeled S194 target cells/well and Con A (10 μg/ml final concentration) were added. Plates were centrifuged for 1 min at 1,000 rpm. and incubated for 4 h at 37°C. Specific isotope release into the medium was determined according to Brunner et al. (24). The same procedure was used for testing cells from cloning plates, except that these were not counted or serially diluted.

Cloning of Cytolytic Hybrids. Seven independent hybrids were cloned by placing an average of one cell/well/200 μl of culture medium supplemented with 25% CS into flat-bottomed 96-well plates, which, in addition, contained 10^4 irradiated (2,000 rad) murine macrophages from a peritoneal wash. The cloning efficiency in this first cloning ranged from 25 to 100%. Growing clones were detached and tested for cytolytic activity in the same way as the original hybrids. Cytolytically active clones were obtained from six of the seven hybrids and all but one cloned hybrid (15.4) were recloned once more. In this recloning step, the cloning efficiency was between 80 and 100%. One clone, 9.4, was also recloned by micromanipulation of single cells into cloning wells.

Selection of CS-independent Variants. Variants were selected in flat-bottomed 96-well microtiter plates containing 10^4 irradiated (2,000 rad) murine peritoneal macrophages, which effected the removal of dead cells by phagocytosis. TCGF-independent, cloned hybrids were washed, resuspended in normal culture medium, and added to the plates at 2 × 10^5-2 × 10^6 cells/200 μl/well. Cells were fed weekly, and after a culture period of 14-18 d, wells containing growing colonies or surviving cells were harvested and assayed for cytolytic activity. From each hybrid some CS- variants were isolated and maintained in normal culture medium in petri dishes.

Determination of VV Resistance, TCGF Dependence, and Titration of CS on Different Cell Lines. For these assays, 10^4 cells/well/ml were placed in 16-mm, 24-well plates and cultured for 4 d. Cells from individual wells were then counted with a coulter counter. To determine their VV resistance, the cells were grown in serial dilutions of purified VV lectin (obtained from J. P. Mach, prepared as described [17]) and 25% of CS. TCGF dependence was assessed by washing the cells and culturing either in culture medium or in medium supplemented with 25% of CS. The relative dependence of cell lines was determined by culturing the washed cells in serial dilutions of CS.

Determination of TCGF Receptors. The capacity of different cell lines to remove TCGF from CS was measured by absorption. The relative amount of TCGF present in CS was measured by its capacity to stimulate growth of the TCGF-dependent CTL line BD2.118, a line developed in our laboratory that grows in suspension. Aliquots containing 4 × 10^5 cells/100 μl were added to serial dilutions of a test sample in 96-well round-bottomed microtiter plates. After 48 h of culture and a final [3H]thymidine pulse of 6 h (1 μCi/well), cells were transferred and washed on glass filters with an automatic cell harvester, and [3H]thymidine incorporation was determined in a β-counter. Plateau levels of incorporation reached 5-10 × 10^4 cpm, whereas control values in the absence of CS were 200-300 cpm. A 1:8 dilution of CS in culture medium was used in absorption experiments, as this was the lowest concentration giving plateau values of incorporation. To remove TCGF from cells grown in CS before the absorption procedure, exponentially growing cells were harvested, washed three times, resuspended, cultured for 6 h in normal culture medium, and then washed once more. Cells grown without CS were simply washed. Sometimes, washed cells were fixed in 2% glutaraldehyde for 5 min at room tempera-
ture, washed, and frozen. For absorption, serial dilutions of washed cells in culture medium were added to round-bottomed 96-well microtiter wells and cooled to 4°C with occasional shaking. Previous absorption studies showed that half-maximal absorption was reached within 5 min. After centrifugation, the supernatants were removed, irradiated (2,000 rad), and frozen, and the remaining TCGF activity was measured as described above. Duplicate samples were tested in both the absorption and TCGF assays, and the percent unabsorbed TCGF was calculated by comparison with the CS titration curve, which had been linearized by probit plot as described by Smith (25).

Cell Surface Labeling with NaB\textsubscript{3}H\textsubscript{4}. Cells were labeled using a procedure essentially identical to the one described by Gahmberg et al. (26). All cells to be labeled were grown for 3 d in culture medium supplemented with 25% CS, regardless of their dependence on CS. Exponentially growing cells were washed, incubated at room temperature for 10 min in 50 mM α-methylmannoside in PBS to remove residual Con A, and then washed three times, resuspended to 10⁷ cells/ml in Dulbecco's PBS, and incubated with galactoseoxidase (5 U/ml) and neuraminidase (0.025 U/ml) for 60 min at 37°C. After an additional three washes, the cells were resuspended to 10⁷/ml and reduced with 1 mCi/ml of NaB\textsubscript{3}H\textsubscript{4} for 40 min. They were then washed twice more and lysed in 0.5% NP-40 plus 1 mM PMSF for 30 min on ice and frozen at −70°C. Alternatively, cells were oxidized with sodium periodate according to Gahmberg and Andersson (27). In this case, washed cells were resuspended to a concentration of 10⁷ cells/ml in PBS, treated with periodate at a final concentration of 1 mM on ice for 5 min, and then washed and reduced as cells oxidized with galactoseoxidase. After addition of sodium dodecyl sulfate, the lysates were boiled for 3 rain as described previously (17), and gel electrophoresis was performed in 8% polyacrylamide slab gels according to Laemmli (28). Gels were processed by fluorography as described by Bonner and Laskey (29).

Determination of the Cellular DNA Content. Exponentially growing cells or freshly isolated mouse thymocytes were washed, and chicken erythrocytes were added to each sample as an internal control. The mixture was then treated with RNase (80 Kunitz U/ml) for 1 min and stained with the DNA-binding dye propidium iodide (50 μg/ml) in the presence of 0.05% NP-40 as described by Taylor and Milthorpe (30). The cells were then passed on a FACS-II flow cytometer (B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) using 488-nm excitation wavelength.

Materials. Plastics were obtained from Nunc (Bio AG/Gibco Europe, Basel, Switzerland); fetal calf serum from Seromed GmbH (Munich, FRG); Con A from Pharmacia Fine Chemicals AB (Uppsala, Sweden); thioguanine, α-methylmannoside, phenylmethanesulfonyl fluoride, RNase, and propidum iodide from Sigma Chemical Co. (St. Louis, MO); hypoxanthine, EDTA, and 2′deoxyxymidine from Merck & Co. (Rahway, NJ); polyethylene glycol, ouabain, amethopterin, and sodium dodecyl sulfate from Serva Feinbiochemica GmbH (Heidelberg, FRG); galactose-oxidase from Kabi AG (Stockholm, Sweden), Nonidet P-40 from Dr. W. Koob AG (Hedingen, Switzerland); 51Cr from Institut National des Radioelements (Fleurus, Belgium) Belgium; and all other radiochemicals, including a 14C-methylated protein mixture, from Amersham International plc (Amersham, England).

Results

Description of Hybrids. All results reported here were obtained with hybrids from a fusion called T2. In this cross, a thioguanin- and ouabain-resistant mutant of the murine T lymphoma BW5147 was crossed with the murine CTL-B line B6.1 SF.1 (derived by von Boehmer et al. [21]) and hybrids were selected in HAT and ouabain. Selecting in CS, we observed colonies in 36 of 48 wells, without CS in 2 of 48 wells. No colonies were observed in a mock fusion without polyethylene glycol. To confirm the hybrid nature of the isolates, we exploited the fact that the two parental lines carry different Thy-1 alleles. Using monoclonal antibodies against Thy-1.1 and Thy-1.2, we found that all isolates expressed both of these determinants. Some hybrids were screened for cytolytic activity, and 7 out of 10 selected in CS had strong activity.
The active hybrids were cloned. Hybrids are named by a series of numbers, the first
of which indicates the original well from which the hybrid was isolated, the second
and further numbers used for clones and subclones obtained from the original isolate.
Thus, hybrids with different first numbers are derived from independent fusion events.
Clones derived by micromanipulation are indicated by the letter M. The phenotype
of the hybrid clones studied further was tested on day 88 after fusion and is described
in Table I. All of them display cytolytic activity, are sensitive to VV, and depend on
CS for growth.

Selection and Properties of CS- Variants. 91–96 d after fusion, i.e., 26–30 days after
cloning, cells of the various hybrids were transferred into medium without CS and
plated at different cell concentrations (2 × 10^4 to 20/well) in microtiter plates. After
10–14 d, colonies of CS- variants from four hybrids became visible to the naked eye.
Assuming 100% cloning efficiency, we calculated the proportions of CS- variants (V)
present at the beginning of the selection using Poisson statistics, and from this figure
and the estimated number of generations (n) elapsed between cloning and the
beginning of variant selection, we determined the rate of variant production (f) by
the equation f = V/n (31). Values for f are 3 × 10^-5 (clone 9.4.7), 6 × 10^-5 (clone
15.4), 7 × 10^-6 (clone 16.8.15), and 1.3 × 10^-4 (clone 25.2.16). Clone 23.6.9 did not
yield CS- variants in two experiments: in this hybrid, f was therefore <4 × 10^-6.
These calculations neglect the fact that CS- variants and CS+ hybrids have not
necessarily the same growth rates and CS- variants may be selected for inadvertently,
as TCGF might become limiting occasionally before subculture. They also do not
take into account fluctuation of the number of variants due to the random sampling
when cultures are split for routine transfer.

Hybrids 9.4.M3 and 33.4.14 did not give rise to distinct CS- colonies, but the
plated cells seemed to remain alive and, after subculturing the content of several wells
with the highest cell density (2 × 10^6), we obtained cell populations that grew in the
absence of CS from both of these hybrids. CS- variants were designated by the
addition of the number of the isolate preceded by the symbol CS to the number of
the parental hybrid. The variants were not cloned and different variants isolated from
the same hybrid are not necessarily independent.

| Table I  |
|----------|
| Characteristics of Cloned Hybrids |
| Cell line | Cytolytic* activity | VV resistance | CS dependence |
|  | 1:1 | 0.3:1 | 50% | 90% | +CS | -CS |
| B6.1.SF.1 | 80 | 60 | 1.1 | 3.2 | 66 | 0.5 |
| BW5147 | --- | --- | >154 | >154 | 77 | 136 |
| 9.4.M3 | 44 | 30 | 1.9 | 5.8 | 50 | 2.3 |
| 9.4.7 | 45 | 33 | 1.9 | 6.3 | 42 | 1.2 |
| 15.4 | 39 | 24 | 2.1 | 9.2 | 41 | 1.6 |
| 23.6.9 | 24 | 12 | 1.9 | 5.8 | 44 | 0.9 |
| 25.2.16 | 19 | 12 | 2.5 | 6.5 | 66 | 1.5 |
| 33.4.14 | 22 | 12 | 1.9 | 6.5 | 39.5 | 0.4 |

* Percent specific 51Cr release at 1:1 and 0.3:1 CTL/target cell ratios.
† Concentration of VV (μg/ml) that reduces cell recovery by 50 and 90%.
§ Ratio of the number of cells recovered after 90 h of culture to the number of cells put into culture at time
0 either with (+) or without (−) CS.
Fig. 1. Cytolytic activity of CS+ clones (●) or CS− variants (○). Contents of wells containing visible colonies were harvested at 14-18 d after plating in microtiter plates. Cells were assayed without counting on S194 target cells in the presence of Con A.

**Table II**

| Cell line | Parental hybrid | CS− variants | CS+ subclones |
|-----------|-----------------|--------------|---------------|
| 9.4.M3    | 60              | 0, 0, 1      |               |
| 9.4.7     | 61              | 0, 0, 0, 6   | 41, 37        |
| 15.4      | 51              | 0, 0, 0, 0   |               |
| 16.8.15   | 39              | 0, 0, 0, 0   | 65, 66        |
| 25.2.16   | 33              | 0, 0, 0, 0   | 35, 35        |
| 33.4.14   | 45              | 0            | 52            |

* Numbers indicate percent specific ¹¹Cr release at a 10:1 cytolytic/target cell ratio.

14 and 18 d after initiation of the selection, growing colonies or surviving cells (9.4.M3) were collected from the selection plates and their cytolytic activity was measured in a semiquantitative assay. As shown in Fig. 1, CS− variants had relatively low cytolytic activity in comparison with subclones isolated simultaneously in CS-supplemented medium. The remaining cytolytic activity of CS− variants might be due to the persistence of CS+ cytolytic hybrids in the selection plates, because 1 wk later, after having been transferred to 60-mm culture dishes, none of the CS− variants had any detectable cytolytic activity, whereas the parental hybrid clones, which had been grown in CS during the whole duration of selection, maintained their lytic capacity (Table II). 28 or 61 d after the beginning of the selection of CS− variants, the hybrids and variants were tested for dependence on CS and VV resistance (Table III). CS− variants were CS independent, whereas all parental hybrids still depended on CS, except 33.4.14. In this hybrid, a high proportion of CS− variants appears to have accumulated at the time of testing (day 61 after the beginning of selection).

Fig. 2 shows the growth inhibition of two hybrids and its variants by different doses of VV. Similar titration curves served as basis for calculations of the sensitivity to VV of the other hybrids (Table III). All CS− variants are highly resistant to VV with the exception of 9.4.M3.CS69, in which, as discussed later on, CS+ cells probably were not completely eliminated by the selection procedure.

Stability of the Phenotype of CS− Variants. After the CS− variants had been maintained in CS-free culture medium for 4 wk, we tested whether their phenotype would revert
by culturing them again in CS for a further 6 wk. Concomitant reappearance of CS dependence, high cytolytic activity, and VV sensitivity was observed only in the case of 9.4.M3.CS69, but not in 9.4.M3.CS52 and two other CS\(^{-}\) variants. The behavior of hybrid 9.4.M3 during the selection of CS\(^{-}\) variants suggests that the reappearance of the parental phenotype in 9.4.M3.CS69 reflected a relative increase of CS\(^{+}\) cells that had escaped selection rather than a change in phenotype of CS\(^{-}\) cells. 9.4.M3.CS69 cells maintained for 6 wk either with or without CS were cloned by micromanipulation. All the clones we obtained were either cytolytic, CS\(^{+}\), and VV sensitive or noncytolytic, CS\(^{-}\), and VV resistant. The latter did not revert to the parental CS\(^{+}\) phenotype during culture in CS. We therefore presume that the 9.4.M3.CS69 population still contained CS\(^{+}\) cells. This is supported by the partial CS dependence and VV sensitivity observed at 28 d after the beginning of selection (Table III). However, the persistence of CS\(^{+}\) hybrids during >30 population doublings in the absence of CS is puzzling. We searched in vain for TCGF activity in the culture supernatants of 9.4.M3.CS69, but it is possible that these cells produce an amount too

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**Table III**

**Phenotype of CS\(^{-}\) Variants**

| Cell line             | CS dependence $\dagger$ | VV resistance $\S$ |
|-----------------------|-------------------------|---------------------|
|                       | +CS     | -CS     | 50% | 90% |
| 9.4.M3                | 40      | 0.7     | 1.8 | 5.5 |
| 9.4.M3.CS52           | 16      | 15      | >154|     |
| 9.4.M3.CS69           | 75      | 38      | 1.2 | 2.3 |
| 9.4.7                 | 62      | 1.2     | 4.8 | 7.8 |
| 9.4.7.CS113           | 11      | 10      | >154|     |
| 15.4                  | 63      | 2.2     | 1.8 | >154|
| 15.4.CS73             | 52      | 57      | >154|     |
| 15.4.CS74             | 53      | 62      | >154|     |
| 15.4.CS146            | 39      | 52      | >154|     |
| 16.8.15               | 52      | 0.6     | 2.4 | 2.5 |
| 16.8.CS116            | 29      | 36      | >154|     |
| 16.8.CS135            | 48      | 52      | >154|     |
| 25.2.16               | 60      | 0.7     | 1.4 | 2.7 |
| 25.2.16.CS77          | 57      | 70      | >154|     |
| 25.2.16.CS142         | 36      | 30      | >154|     |
| 25.2.16.CS143         | 46      | 80      | >154|     |
| 33.4.14$^*$           | 98      | 19      | 2.1 | 22.3|
| 33.4.14.CS82$^*$      | 73      | 79      | >154|     |
| B6.1.SF.1             | 82      | 0.7     | 1.1 | 1.8 |
| BW5147                | 36      | 124     | >154|     |

* Experiments were done 24–28 d after the beginning of the selection except for 33.4.14 and 33.4.14.CS82, which were tested 61 d after the beginning of selection.

† Ratio of the number of cells recovered after 90 h of culture to the number of cells put into culture at time 0 either with (+) or without (−) CS.

§ Concentration of VV (μg/ml) that reduces cell recovery by 50 and 90%.
Fig. 2. VV-resistance of two CS⁺ hybrids and their CS⁻ variants. In the absence of VV, the cell recovery after 4 d of culture in the various cell lines was between 30- and 53-fold higher than the number of cells put into culture. Growth in the absence of VV was taken to be 100%.

Fig. 3. Absorption of TCGF by various cell concentrations. BW5147 (○); B6.1.SF.1 (○); 25.2.16 (▲); 25.2.16.CS142 (△).

low to be detected by the current methods but sufficient to allow survival and slow growth of CS⁺ cells. We cannot exclude the possibility that the 9.4.M3 population contained a class of variants that is either not completely or not irreversibly CS⁻. The occurrence of such variants might be related to the fact that no distinct CS⁻ colonies were observed in the selection plate of 9.4.M3, which indicates that no CS⁻ variants pre-existed in this clone at the beginning of the selection.
Fig. 4. Photomicrographs of 25.12.15 (A) and 25.2.16.CS142 (B).

Fig. 5. Surface glycoproteins of CS⁻ hybrids and CS⁺ variants separated by gel electrophoresis. Cells were labeled by oxidation with galactose-oxidase/neuraminidase and subsequently reduced with NaB₃H₄ at the beginning of CS⁻ variant selection (day 100, F, I) or at day 145 (C, D, E, G, H, K). A, B6.1.SF.1; B, BW5147; C, 9.4.M3; D, 9.4.M3.CS52; E, 9.4.M3.CS69; F, 16.8.15; G, 16.8.15.CS135; H, I, 25.2.16; K, 25.2.16.CS142. 50,000 cpm were loaded in each slot except for B, where 100,000 cpm were loaded. Weight standards indicated at the left side of gel I were ¹⁴C-methylated derivatives of myosin (200 kd), phosphorylase b (92.5 kd), bovine serum albumin (69 kd), and ovalbumin (46 kd). The T130 and T145 gps of BW5147 and B6.1.SF.1, respectively, are indicated by an asterisk. Bands, or groups of bands, of the parental hybrids are designated by letters a-f.

Expression of TCGF Receptors in the CS⁻ Variants. We measured the capacity of hybrids 25.2.16 and 25.2.16.CS142 to remove TCGF from CS. Results obtained with this absorption assay (25) are in accordance with more direct quantitations of surface receptors using purified, radiolabeled TCGF (6). As compared with B6.1.SF.1, the hybrid and its CS⁺ variant have ~2-fold lower capacity to absorb, whereas that of BW5147 is at least 15-fold lower (Fig. 3). Essentially identical results have been obtained with glutaraldehyde-fixed cells (data not shown). Thus, although independ-
ent of TCGF, 25.2.16.CS142 still absorbs TCGF as efficiently as the parental hybrid.

Morphology and Adherence to Plastic of CS+ and CS− Hybrids. B6.1.SF.1 cells adhere to tissue culture plastic as do many other CTL lines in contrast to BW5147 and other T lymphomas. To compare hybrids with regard to this property, CS+ and CS− cells were grown in tissue culture dishes for 3 d in the presence of Con A-free CS. Thereafter, the percentage of cells that could be removed by gentle washes was determined. For CS+ hybrids, the values ranged from 12 to 32%, whereas they were between 85 and 95% for the CS− variants. Thus, the CS+ and CS− hybrids resemble the CTL and thymoma parental cells lines, respectively. Microphotographs of a parental hybrid and a CS− variant derived from it are representative for the

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**Fig. 6.** DNA content of hybrids was measured at day 96, i.e., before the selection of CS− variants was begun (□), or 28 d later (○). Results are given as the ratio of the channel number containing the G1-peak of the test cells to that containing the peak of chicken erythrocytes. Each value is based on the measurement of 3,000 cells. Bars indicate 1 SD from the mean. Open symbols, CS− cells; closed symbols, CS+ cells. Sum of the DNA of the two parental cells (----).
morphological appearance of all the T2 hybrids (Fig. 4).

Surface gp Patterns. CTL lines express T145 but not T130, whereas T lymphomas express T130 but not T145 (17). To compare surface gp of CS* hybrids and CS- variants with those of the parental cell lines, the oligosaccharides at the surface of intact cells were radiolabeled by treating the cells with galactose oxidase and neuraminidase followed by exposure to tritiated borohydride. Parental hybrids were labeled at the beginning of the variant selection (day 100 after fusion) and again 45 d later (day 145) together with CS- variants. Separation of the labeled gp by gel electrophoresis yielded the autoradiographs of Fig. 5. Glycoprotein patterns of different CS* hybrids resemble each other, although band c of 9.4.M3 has the mobility of the T145 of the B6.1.SF.1 line, whereas in the other CS* hybrids we find this band at a position that is intermediate between T145 and T130. Apart from this difference and from differences in the relative intensity of various bands, the parental hybrids have comparable gp patterns. Because VV binds a majority of surface gp of CTL lines (17), and no other specific reagent for the T145 gp is available, we have no means to unambiguously identify T145 in the hybrids, where its mobility is not identical with the T145 of the parental lines. The CS- variants show none of the parental bands a-f of the parental CS* hybrids and express no band with the mobility of the T130 of BW5147. We do not know how the bands of the variants are related to those of the parental hybrids. The difference in surface gp between parental hybrids and variants might reflect either the expression of different gp or different processing (e.g., glycosylation) of the same peptides. A similar change between CS* hybrids and CS- variants is observed when the surface gps are labeled via the sialic acid of their oligosaccharide moieties using periodate (data not shown).

DNA Content of Hybrids and Variants. CS- variants might arise as a result of massive chromosome loss from the parental clones. Therefore, we decided to compare DNA contents of CS* and CS- hybrids by staining cells with propidium iodide. The results (Fig. 6) are given on a scale, on which the loss of an average size chromosome measures 0.08 U. It appears that all hybrids have DNA contents that are close to the sum of the DNA content of the two parental lines. The DNA content of CS- variants is either lower, equal to, or, in one case, higher than the one of the corresponding parental hybrids. Where CS- variants and CS* subclones were derived in parallel, there was no significant difference between them. The biggest difference among parental CS* clones and CS- variants are in the order of 1.2 U, i.e., ~14 chromosomes. Deviations of this size are statistically significant. Coefficients of variance show that the heterogeneity of the cloned CS* hybrids and CS- variants is greater than in the thymocyte standard population but is similar to the one of the parental cell lines.

Discussion

Among CTL × T lymphoma hybrids, the correlation between the continued expression of cytolytic activity and the addition of CS to the medium during hybrid selection has been observed in each of six independent crosses involving two different CTL-B lines (CSP.2.4 [32] and B6.1.SF.1[21]) and three different T lymphomas (BW5147, AKR.A, and MbC12, all derived from AKR mice), as well as in crosses between BW5147 and cytolytic T cells generated in mixed lymphocyte culture or between BW5147 and CTL-A lines (12). We have tested over a hundred hybrids selected in the absence of CS and all of them had no detectable or very low cytolytic
activity and were, as far as tested, VV resistant (33).

We retained the term "CS dependence," as we have not tested whether pure TCGF is able to maintain the growth of CS\(^+\) hybrids. The purification of TCGF has been based on its capacity to stimulate proliferation of CTL-B lines and the parental CTL lines used for the crosses described here can be grown in partially purified human TCGF (1,000-fold purification from leukocyte culture supernatants containing 0.5% serum) (O. Acuto, unpublished observation). Various preparations of TCGF, partially purified from different sources and assayed on different T cell lines have comparable biochemical properties, and there is no evidence to suggest that more than one type of TCGF exists (6, 8, 34-36).

It is unlikely that the CS\(^+\) hybrids are dependent on another factor in CS that is different from TCGF and not required by either parental cell line. The fact that CS contains comparable amounts of growth-promoting activity for CS\(^+\) hybrids and the B6.1.SF.1 parental line is circumstantial evidence against this possibility (Fig. 7).

We find that cloned, cytolytic, CS\(^+\), VV-sensitive hybrids give rise to variants that are CS\(^-\) and VV-resistant and that have drastically reduced cytolytic activity. Phenotypic changes of this type have also been observed in a cross (MN12) between BW5147 and a CTL-B line called CSP.2.4. (33), in which we analyzed CS\(^-\) variants from five CS\(^+\) hybrid clones derived from two independent hybrids.

With regard to VV resistance, the CS\(^-\) variants resemble hybrids selected originally in the absence of CS. For most CS\(^-\) variants this is also true with regard to cytolytic activity. Reinduction of cytolytic activity by culture in CS has not been observed in CS\(^-\) hybrids originally selected in the absence of CS and has been the exception.

![Figure 7](image_url)

**Fig. 7.** Dependence of growth of B6.1.SF.1 and of 25.2.16.M3 on the concentration of CS.
among CS\textsuperscript{−} variants derived from CS\textsuperscript{+} hybrids, as it occurred only in one of four CS\textsuperscript{−} variants of the T2 fusion and was not observed in variants from the MN12 cross (A. Conzelmann, unpublished observation). The CS\textsuperscript{−} variants also show a characteristic surface gp pattern different from the one of CS\textsuperscript{+} hybrids which, in the case of the MN12 cross, is identical with the one of hybrids originally selected in the absence of CS (A. Conzelmann, unpublished observation).

Because, with regard to their phenotype, the CS\textsuperscript{−} variants derived from CS\textsuperscript{+} hybrids and the CS\textsuperscript{−} hybrids originally selected without CS are indistinguishable, we consider the latter as early arising CS\textsuperscript{−} variants of CS\textsuperscript{+} hybrids. This implies that the primary fusion product of a CTL with a T lymphoma line is CS dependent. This hypothesis is also supported by the fact that we consistently found significantly more hybrid colonies when selecting in the presence of CS than without (12).

If the primary fusion product is CS\textsuperscript{+}, but can give rise to CS\textsuperscript{−} variants, we can postulate a genetic element present in the CTL line that represses the TCGF-independent mode of proliferation of the T lymphoma parent. In the simplest case, this may be a gene, switched on in CTL but inactive or lost in AKR-T lymphoma cells, the product of which prevents cell division unless it is inactivated or blocked in its action as a result of the exposure of the cell to TCGF. Loss or inactivation of such a gene in the hybrids would render the cell TCGF-independent. Because three out of four CS\textsuperscript{−} variants from fusions MN12 and T2 (Fig. 4) (M.C. and A.C., unpublished) still express TCGF-receptors, it appears that the gene(s) controlling their expression has (have) to be different from this hypothetical "repressor gene."

The mechanisms of growth control in normal and transformed cells have been studied by means of somatic cell fusion between transformed and nontransformed cells (for review see 37) and both "dominance" as well as "recessiveness" of the nontransformed phenotype have been reported. The contradictory findings might in part be caused by the fact that different parameters such as saturation density, serum requirement, senescence, malignancy, etc., were used to distinguish the normal from the transformed phenotype and that most fusions involved cells differing with regard to species origin, histiotype, and mode of transformation. Our hybrids belong to the class in which the nontransformed growth control regulation is "dominant." We are not aware of another report where a well-defined growth factor dependence has been analyzed in somatic cell hybrids.

A better interpretation of our results may be possible when we understand the way by which the BW5147 thymoma parent is transformed. There is compelling evidence to imply endogenous murine leukemia virus in AKR leukemogenesis. However, the mechanism of transformation in this system is not yet understood (38). Although it is conceivable that some T lymphocyte leukemias respond to TCGF that they produce themselves (39), there is no indication that this is the case for BW5147, as this line does not produce TCGF either constitutively or after stimulation with mitogens (34, 40) and has no or only low amounts of TCGF receptors (Fig. 4). Nevertheless, we have to consider the possibility that TCGF-independent hybrids produce and respond to TCGF. We could not detect TCGF in the spent medium of several independent CS\textsuperscript{−} variants even after stimulation with Con A and phorbol myristate acetate, but we cannot exclude the possibility that TCGF is produced and used at rates that preclude the accumulation of detectable amounts in the medium. If CS\textsuperscript{−} variants are CS independent because they make TCGF, then they have to be able to utilize it
without expressing TCGF-receptors at the surface, as we have found one CS− variant (15.4.CS146) that expresses no detectable absorbing capacity for TCGF (data not shown).

We have proposed chromosome loss as one possible mechanism by which CS− variants arise from CS+ hybrids. The hypothesis predicts that cloned CS− variants cannot give rise to CS+ hybrids. Although we have not observed this type of transition in the limited number of clones derived from 9.4.M3CS69 and 15.4.CS146, we cannot exclude that it occurs at low frequency since we have no powerful selection system for CS+ variants. The comparison of the DNA content of CS+ and their CS− variants allows us to exclude massive chromosome loss as a cause of the appearance of the CS− phenotype. To obtain conclusive evidence for or against models implying different chromosome constitutions of CS+ and CS− hybrids, it will be necessary to reproduce our findings in crosses where parental chromosomes can be karyotypically distinguished.

Alternative explanations for the transition from the CS+ to the CS− state imply regulatory events involving a change in the state of the genetic element of CTL lines, which, in the hybrids, represses the CS independent replication of AKR-thymomas. It is difficult to build experimentally testable models of this kind, but it is worth noting that we could not obtain CS− variants from parental CTL lines (M. Nabholz, unpublished observations).

The maturation of most CTL-precursors into mature, cytolytically active cells involves the acquirement of TCGF-dependence. We do not know whether the coordinate expression of cytolytic activity, GS dependence, and VV resistance that was observed in several independent crosses between CTL lines and T lymphomas is related to a regulation mechanism that is governing normal T lymphocyte maturation. CS− hybrids might fail to lyse target cells for fortuitous reasons. They might not, e.g., properly interact with Con A, which is needed in the CTL assay as the T2-hybrids, unlike the parental B6.1.SF.1 CTL line, have no activity in its absence. This possibility is however unlikely, as CS− variants from other crosses were noncytolytic in spite of the fact that the parental hybrids were killing specifically.

On the other hand, a genetic element that represses a whole set of liver-specific functions has been found in dedifferentiated rat hepatoma lines (41, 42). An analogous genetic element might for instance be present in BW5147, become inactive upon fusion with a CTL line and become activated again in the CS− variants.

Summary

Somatic cell fusion between cytolytically active, T cell growth factor- (TCGF) dependent murine T cell lines (CTL lines) and noncytolytic, TCGF-independent murine T lymphoma lines has yielded two types of somatic cell hybrids (5): cytolytic hybrids, growth of which is dependent on TCGF, and hybrids with very weak or undetectable cytolytic activity which grow at the same rate with or without TCGF. Here we report that the former can produce stable variants that resemble the latter type. Some of these TCGF-independent variants still have TCGF receptors. High susceptibility to the cytotoxic effects of *Vicia villosa* lectin, a marker distinguishing the parental CTL lines from T lymphomas, is expressed by the TCGF-dependent hybrids.

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3 A. Glasebrook. Cytolytic T lymphocyte clones which proliferate autonomously in response to antigenic stimulation. Manuscript submitted for publication.
but not by the TCGF-independent variants. The two types of hybrids also differ in the expression of surface glycoproteins. We propose that there exists a genetic element in the CTL line that represses the TCGF-independent replication mechanism of the T lymphoma parent in the TCGF-dependent hybrids and that this genetic element is lost or switched off in the TCGF-independent variants.

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