PRODUCTION, EXPANSION, AND CLONAL ANALYSIS OF T CELLS WITH SPECIFIC HLA-RESTRICTED MALE LYSIS*

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The possibility of maintaining human T cells in vitro for a prolonged period of time has been known for some years (1). Human cytotoxic T cell (CT)[1] lines grown as a population (CT lines) can be maintained in culture in various ways. Several investigators have reported the use of conditioned medium (CM) or T cell growth factors (TCGF) (2–10). Others used mitogens for expanding and maintaining CT lines (11–13). Based on these experimental data, we investigated whether anti-H-Y HLA-A2-restricted effector cells could be cultured, expanded, and cloned.

Materials and Methods

Source of Human Lymphocytes. Peripheral blood lymphocytes (PBL) were obtained from a female patient who was suffering from aplastic anaemia, in partial remission. (HLA-phenotypes A2, Bw44, B40, Cw3, Cw5, Dw4, Dw6, DRw4, DRw6) (14, 15). The lymphocytes were separated from her peripheral blood by Ficoll-Isopaque (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) -gradient centrifugation. We have shown previously that her cells (after a 6-d in vitro sensitization against the irradiated PBL from an HLA-A, -B, -C, and -DR-identical but mixed lymphocyte reaction [MLR]-positive unrelated male donor) were able to lyse cells from HLA-A2-positive male donors but not cells from other donors, with a few exceptions (15).

Growth of the Cytotoxic Anti-HLA-A2 H-Y T Cell Line

PRIMARY CULTURE PHASE AND LONG-TERM GROWTH. For the primary sensitization (i.e., primary culture), the antigen-specific CT cells were cultured with an equal number of irradiated stimulator cells (HLA-A, -B, -C, and -DR-identical, but MLR-positive, unrelated male donor) for 6 d at 37°C in tissue culture flasks (3013; Falcon Labware, Div. of Becton, Dickinson & Co.,

* Supported in part by the Dutch Organization for Health Research (TNO), the Dutch Foundation for Medical Research (FUNGO), which is subsidized by the Dutch Organization for the Advancement of Pure Research (ZWO), and the J. A. Cohen Institute for Radiopathology and Radiation Protection (IRS), and by the Institut National de la Santé et de la Recherche Médical (CRL 79.5.118.1; CRL 79.5.088 1 and AU 78.150) and the Délégation Générale à la Recherche Scientifique et Technique (DGRST) (ACC 76.7.0966 and ACC 77.7.1370).

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Abbreviations used in this paper: CM, conditioned medium; CML, cell-mediated lympholysis; CT, cytotoxic T cell(s); CT lines, cytotoxic T cell line(s) grown as a population; Dil, limiting dilution(s); E/T, effector:target [ratio(s)]; [^{3}H]TdR, [^{3}H]thymidine; LU, lytic unit(s); MLR, mixed lymphocyte reaction; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; TCGF, T cell growth factor(s)

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Volume 152 August 1980 182s–190s

Oxnard, Calif) kept at an angle of 45° in a humidified 5% CO_2 incubator The culture medium was RPMI-1640 that was supplemented with 20 mM L-glutamine/ml (Flow Laboratories Ltd, Irvine, Scotland) plus 20% heat inactivated pooled human AB serum from healthy male donors After 6 d of vitro sensitization, 3×10^6 effector cells were taken from the primary culture to initiate and maintain the first CT line Irradiated feeder cells (3,500 rad) from the specific stimulator (0 5 cells $\times 10^6$/ml), and phytohemagglutinin (PHA) (670 0576, 1 100 final dilution, Gibco Diagnostics Laboratories, Gibco Invenex Div, Lawrence, Mass) were used throughout this study (12, 13) The CT lines were maintained and expanded by this procedure for 68 d Each CT line was regularly tested for specific proliferative response and cytotoxic capacity against the appropriate target cells Using the same techniques, five different CT lines were initiated from the primary culture

CLONING Repeated limiting dilutions (Dil) from the first CT lines were made in such a way that, statistically, one would expect only either 10 cells or 1 cell per conical microtiter well (250 μl vol) Growth promotion was provided by adding γ-irradiated feeder PBL and the lectin PHA to the wells Usually, the 10-cells/well dilution resulted in 100% growing colonies, whereas with 1-cell/well dilution, growing colonies were observed in 20–30% of the wells after 10–20 d of culture without any change of medium

We started by using Dil both at 10 cells and 1 cell per well, colonies obtained from the first Dil are refered to as primary colonies Feeder cells (1×10^4) and PHA (1 100 final dilution) were added to each well, as described above [2] After 10 d, the content of each well was transferred separately to flat-bottom microtiter wells (250 μl vol) with addition of irradiated feeder cells (3×10^4) and PHA After 10–15 d with no change of medium, each well was screened for the presence of growing colonies If such colonies were found, the cells from one positive well were transferred to 2 ml cluster wells (3524, Costar, Data Packaging, Cambridge, Mass) Culturing was continued using feeder cells and PHA as described above As soon as enough cells could be harvested, they were tested for cytotoxicity In this way, 68 separate cultures were grown

Dil I was derived from one of the first CT lines in dilutions of 10 and 1 cells per cell, and was grown as described above, we will refer to these as primary colonies

Dil II was derived from Dil I 72 wells that originally contained only 10 cells per well were pooled and were diluted to 10 cells per well and was grown as described above

Dil III has been derived from only one 10 cell well from Dil I that lysed the HLA A2 positive male cells Dil III has been diluted to 10 cells per well and was grown as described above We will refer to these as cloned cultures

Cell-mediated Lympholysis (CML) Assay The CML assay used has been described previously (15) Briefly

(a) Each effector-cell combination to be tested (e g, CT lines, primary colonies, and cloned cultures) was washed, resuspended in fresh culture medium and, counted

(b) Target cells were PBL stimulated with PHA M (0528-57, Difco Laboratories, Detroit, Mich) for 72 h and labeled thereafter with 100 μCi of ^{51}Cr (CJS 1P, $Na_2 CrO_4$, 100 350 mCi/mg) (Amersham, Buckinghamshire, England) for 1 h at 37°C After washing, suspensions of 10^6 target cells/ml were made and used constantly The effector and target cells were mixed (at varying effector target [E/T] ratios) in round-bottom microtiter plates and incubated for 4 h at 37°C After the incubation time, the microtiter plates were centrifuged for 10 min at 500 *g*, the supernates were removed with the Flow supernate harvester system (Flow Laboratories, Inc, Rockville, Md) The samples were counted in a γ-counter (5260 (Packard Instrument Co, Downers Grove, Ill)), in most instances, triplicate wells were tested in at least six effector cell dilutions

Spontaneous release values were derived from those wells with target cells incubated in culture medium alone Maximum release values were obtained from Zaponin- (Zaponin, Coulter Electronics Ltd, Herts, England) distilled-water lysis of the target cells

(c) The percent lysis was calculated using the following formula

[2] Malissen B, D Charmot, and C Mawas Expansion of human lymphocyte populations expressing specific clones either cytotoxic or proliferative, obtained from a population of responder cells primed in vitro Manuscript submitted for publication

$$\text{percent lysis} = \frac{\text{counts per minute experimental mean} - \text{counts per minute spontaneous release mean}}{\text{counts per minute maximum release mean} - \text{counts per minute spontaneous release mean}} \times 100\%$$

The data are expressed on a scale on which 0% was made equivalent to the spontaneous release value and 100% to the maximum release value Standard errors of mean of the triplicates were always <5% Percentages ≤10% were considered negative when only one E/T ratio was used Some data are expressed in lytic units (LU) LU 30/50 for example is the number of effector cells × 10^{-4} necessary to obtain 30/50% specific lysis of 10^4 target cells According to this definition, a larger LU size reflects a weaker response because more effectors are required to cause a 30 or 50% CML response

Proliferative Response This was done essentially as described earlier (12, 13) Briefly 10^4 responder cells (population) were incubated with 10^5 x-irradiated stimulator cells for 4 d [^{3}H]thymidine ([^{3}H]TdR) was added at day 3, and the culture was continued for 16 h Harvesting and counting procedures have been described in detail elsewhere (12, 13)

HLA-A, -B, C, and -DR Typing This was done according to standard techniques described by van Rood (16) and van Rood et al (17)

Surface Markers E rosettes were prepared using papainized sheep erythrocytes Contamination with B cells and/or monocytes was checked by labeling the cells with FITC-anti-Ig immunoglobulin as described by van Leeuwen (18)

Results

Membrane Characteristics of Cloned Cultures Table I summarizes the results obtained in the characterization of two of the Dil III cloned cultures The data show that the cell population consists mainly of T cells Furthermore, the cells express the HLA-A, -B, and -C antigens of the original PBL on their surface Typing for HLA-DR of the cloned T cells shows expression of the same alleles as on the original PBL More than 80% of the cells express DR antigens Thus, CT lines in their active state do express the HLA-DR antigens, although formal proof (i e inhibition of cytotoxicity by anti-HLA-DR antisera) has yet to be shown

Cytotoxic Characteristics of the Expanded CT lines, the Primary Colonies, and the Cloned Cultures Several CT lines were regularly tested in CML against four different HLA-A2-positive and four different HLA-A2-negative male target cells, three different HLA-A2-positive and two different HLA-A2-negative female target cells Fig 1 shows

TABLE I

Characteristics of Cloned T Cell Cultures

	Surface Ig	E rosettes	Cytotoxic scores with anti HLA sera			Cytotoxic scores with anti HLA A, B C sera						
			DRw4	DRw6	DRw1, 2, 3, 5, 7 8	A2	Bw44	B40	Cw 3	Cw5	w4	w6
	%	%										
Dil III clone No 9	1	85	1	4	0	5	5	5	4	4	5	5
Dil III clone No 19	3	92	4	4	0	ND	ND	ND	ND	ND	ND	ND
PBL	20	70	5	4	0	5	4	5	5	4	5	5

The cytotoxic scores with the anti HLA A, B, C, and DR sera are as follows 5 = 100% cytotoxicity, 4 = 80% cytotoxicity, 0 = no cytotoxicity observed, ND, not done

that the expanded cells of three different CT lines, collected on day 22 after initiation of the cell culture (day-22 cells), exert a selective and strong cytolytic activity against the HLA-A2-positive male target cells. The different CT lines derived from the primary culture showed a maximum of ~80% lysis at an E/T ratio of 10:1, with only a slight increase at larger E/T ratios. Fig. 1 shows three of the five tested CT lines; all showed the same pattern.

The first Dil (Dil I) was made from one of the CT lines. From Dil I, we were able to grow primary colonies. Because we were confronted with a large number of such

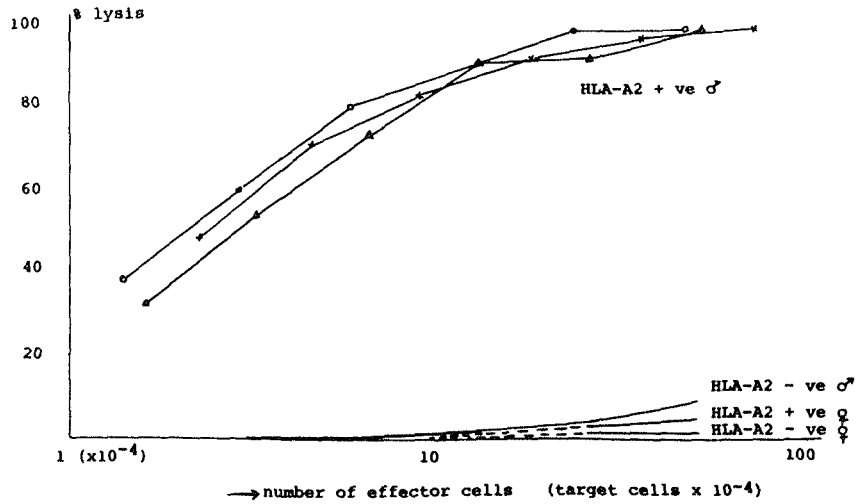

FIG. 1. Specific anti-HLA-A2 H-Y lysis by CT lines (day 22). The levels of cytotoxicity expressed by three expanded CT lines (day 22) against HLA-A2-positive male cells are compared to the absence of lysis observed against HLA-A2-negative males and HLA-A2-positive or -negative females.

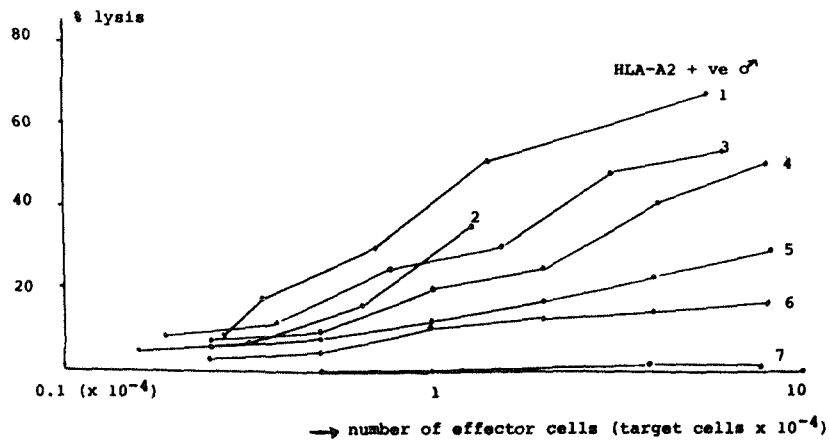

FIG. 2. Lytic capacities of some primary colonies derived from 1 or 10 cells originally. 7 of the 15 primary colonies tested against an HLA-A2 male target cell are shown. The LU are expressed in numbers of cytotoxic cells × 10^{-4} target cells required to give 30% specific lysis. Lines 1–7 are derived from 10 cells/well Dil, except line 3 which contained originally only 1 cell/well. Primary colony 1: 0.7 LU, 2: 1.1 LU, 3: 1.6 LU, 4: 2.5 LU, 5: 9.6 LU, 6: >100 LU, 7: >100 LU. Titration curve of primary colony 1: E/T = 5.6:1, +67% lysis; ET = 2.8:1, +59% lysis; E/T = 1.4:1, +51% lysis; E/T = 0.7:1, + 30% lysis; E/T = 0.35:1, +18% lysis; and E/T = 0.17:1, +9% lysis.

TABLE II

Cytolytic Capacity of 24 Primary Colonies Derived from Original 10-Cell Populations and Thereafter Recloned by Limiting-Dilution

	Percent lysis (HLA-A2 + ve ♂)			
	<10%	10–30%	30–50%	>50%
Number of primary colonies	13	2	4	5

The 24 primary colonies were all tested in E/T ratios between 1 1 and 10 1, except four of the negative ones which were tested at a higher E/T ratio

TABLE III

*23 Clones Tested for Cytotoxicity against an HLA-A2-positive Male and Female Target Cell**

Clone number	E/T ratio	Target (A2 + ve ♂)	Target (A2 + ve ♀)
1	19 1	+22	0
2	13 1	+27	−1
3	10 1	+7	−1
4	16 1	+26	−2
5	11 1	+36	−1
6	21 1	+32	−1
7	11 1	+26	0
8	10 1	+19	0
9	11 1	+39	−1
10	14 1	+31	0
11	24 1	+29	−1
12	20 1	+30	0
13	10 1	+27	−1
14	11 1	+24	0
15	20 1	+22	0
16	16 1	+26	−1
17	23 1	+33	−1
18	14 1	+34	+1
19	17 1	+20	−1
20	31 1	+32	+1
21	15 1	+42	+1
22	20 1	+34	−1
23	11 1	+34	0

* Dil III (23 wells) derived from 1 well (10 cells) from Dil I

primary colonies we have screened only few of them. On the 38th d after initiation of the culture, we screened 15 primary colonies at six different dilutions on several target cells (males or females) with different specificities for HLA and H-Y.

Fig. 2 shows the percentages of lysis capacity of 7 of the 15 primary colonies tested, which were derived by Dil (Dil I) Positive lysis occurred only with HLA-A2-positive male targets cells. The data show great variation in cytolytic capacity, which we will refer to as the fan-shaped phenomenon. Some primary colonies are able to induce 50% lysis at a 1:1 E/T ratio, whereas others show much less lytic potential (including a negative one). Furthermore, the lytic capacity of the different primary colonies are also expressed in LU. All the primary colonies have been tested at six E/T ratios (Fig.

TABLE IV
[^{3}H]TdR Uptake by Day 31 of Several Anti-HLA-A2 H-Y CT Lines

Responder cells,* CT line	Stimulator cells	
	A2 + ve ♂	A2 − ve ♂
1	147‡	2,514
2	96	831
3	81	786
4	60	468
5	63	771

* 10^4 responder cells and 10^5 x-irradiated stimulator cells were mixed in conically bottomed microtiter plates [^{3}H]TdR incorporation was measured by an overnight pulse at day 3 for 16 h
‡ Results are expressed as mean counts per minute of triplicates

2). The titration of the line 1 primary colony against the HLA-A2-positive male target cell is indicated (Fig. 2).

The primary colonies obtained from Dil II (a pool of the cells of 72 wells that originally contained 10 cells derived from Dil I (Materials and Methods) were tested for cytotoxicity The results of 24 colonies are expressed in percentages of the cytotoxic capacity obtained against an HLA-A2-positive male target cell and are shown in Table II. Again the fan-shaped phenomenon occurred. Table III shows the results of the cloned cultures from Dil III, derived from only one cytotoxic-positive primary colony from Dil I Only 1 cloned culture out of 23 is negative against HLA-A2-positive male target cells. The other cloned cultures, as expected (because Dil III had been made from one cytotoxic-positive well), showed specific HLA-A2-restricted anti-H-Y lysis, but in this case, with little variation in the intensity of the lysis from one clone to the other.

Proliferative Study of the CT Lines toward Male Cells. Apart from the study of the cytolytic capacity of the various effectors derived in this study, proliferative assays were also carried out in parallel. CT lines were tested for their proliferative response. Table IV shows that the highly cytotoxic effector-cell populations gave much lower incorporation of [H^3]TdR against HLA-A2-positive male stimulator cells than against HLA-A2-negative male cells.

Discussion

In an earlier work, Goulmy et al. (19) have shown that from aplastic anemia patients who expressed the H-Y HLA-restricted phenomenon toward more than one HLA specificity, cytotoxic T cells could be separated; both on the basis of specific monolayer absorptions (19), as well as cold-target inhibition studies (E. Goulmy. Unpublished data.), suggested a clonal expression of the cytotoxic effectors. Recently, after the developments in human T cell expansion (1–13) as well as cloning (20),[2] we attempted to demonstrate directly that, indeed, cloned cultures of H-Y HLA-restricted T cells could be derived, maintained, and expanded for prolonged period of time from populations that contained anti-H-Y CT.

In this report, we show that it was possible to derive five populations of CT lines that expressed the original lytic specificity from PBL of a female aplastic anemia patient known to express mainly male HLA-A2-restricted T cell-mediated lysis. Those

five different CT lines were expanded using as growth promoter an irradiated feeder (PBL) and a lectin (PHA) as described previously (11–13). The cytotoxic characteristics of the five CT lines were identical both in terms of specificity (HLA-A2 H-Y restriction) as well as in their lytic potential (three CT lines are shown in Fig. 1). None of these five CT lines were able to show specific [H^3]TdR incorporation against their target cells or any detectable proliferation (in terms of cell numbers). The CT lines, as well as the primary colonies, can be expanded, therefore, only in the presence of a growth promotor provided in this study, i.e., solely through the use of feeder cells (γ-irradiated PBL) and a lectin. This confirmed one of the major characteristics of T cell lines both in mice and men, i.e., their absolute requirement for a growth promoter (whether CM or, as in our experience, lectin plus irradiated feeder cells). All the primary colonies tested, as well as the CT lines have been grown to 10×10^6 cells and then frozen. Thawing of the CT lines or the primary colonies and further expansion with the functional reactivity is possible (data not shown). Five CT lines have maintained both their cytotoxic specificity as well as their growth potential over a period of 68 d.

In parallel to the generation of CT lines that express the original H-Y HLA-A2-restricted cytolysis, attempts have been made to derive clones from the CT lines. To do so, we used the Dil technique; colonies obtained are refered to as primary colonies. From Dil I, 10 out of 15 primary colonies tested showed specific cytotoxic activity (7 examples are shown in Fig. 2) in a fan-shaped pattern. Furthermore, the cytotoxic capacity seemed to be increased by cloning. A marked difference was even observed after the first Dil. Expressed in LU: CT line 1 had a cytotoxic capacity of 2.4 LU 50, whereas primary colony No. 1 showed 1.4 LU 50. (1 LU 50 is the number of effector cells necessary to obtain 50% specific lysis.)

The fan-shaped phenomenon can be explained in at least three ways. First, it could be a reflection, at the clonal level, of heterogeneity of the T cell receptors expressed by each individual clone exerting varying affinities. Second, it might be a result of an unequal lytic potentiality of each clone. To test for these two alternatives, we intend to take advantage of the use of a lectin, like PHA or concanavalin A, during the CML assay, because lectins increase cytotoxic capacity (21). Finally, the differences observed in lytic potential between the individual primary colonies could merely be a result of differences within the cloned cell cultures in terms of growth synchrony. This also could be tested using appropriate synchronization techniques.

Up to this point we have referred to primary colonies. We are aware that to speak of clones, it is necessary to qualify the term unambiguously. In the absence of the precise tools that would allow one to characterize the products of monoclonal-antibody-secreting hybridomas, we have refered to primary colonies without an unambiguous definition of this term. We have therefore done new Dil from one original primary colony. In such an experiment, a third Dil from a primary cytotoxic colony gave an almost perfect fit with the expected results, as shown in Table III. Despite the fact that the clones were derived from 10-cell wells, we feel confident that one could umambiguously reach the stage of true cloning without the use of more sophisticated techniques.

Among the primary colonies obtained which are not cytotoxic, we have not yet been able to check whether they could express some other biological functions. One obvious function to look for is the helper activity (22, 23).

Finally it is of interest to note that as far as cell markers are concerned, the cloned CT seem to express identical markers as those reported on expanded human T cell populations (5, 24, 25) The cultured colonies tested are Ig negative, E-rosette positive, HLA-A, -B, and -C positive, and also HLA-DR positive (>80% of the cells from each cultured colony are DR positive) The DR alleles found on the T cells are identical to those found on B cells from PBL and on lymphoblastoid cell lines derived from the same individual (unpublished data)

These data show that both CT lines and cloned cells can be derived from an in vivo immunized woman and that both the population and the cloned cells can be expanded while their specific alloreactive HLA-restricted anti-male cytotoxic reactivities were maintained

It is possible to culture such cells to a number required for the biochemical studies of membrane-specific markers and/or receptors Of special interest would be analysis of the cloned human anti-HLA-A2 H-Y T cell receptor

Summary

A cytotoxic T cell (CT) lines grown as a population (CT line) was initiated from the peripheral blood lympocytes (PBL) of a female aplastic anemia patient who was known to express CT that were able to lyse HLA-A2-positive male cells The anti-H-Y HLA-A2-restricted cytotoxic activity could be maintained over prolonged periods of time The CT lines could be expanded and maintained in culture for >65 d by the use of mitogens and irradiated feeder cells Out of 68 cultures obtained after cloning of the CT lines, 43 showed varying, but always specific, anti-H-Y HLA-A2-restricted lytic capacity on a per-cell basis We could show that the cloned cultures were composed of >80% T cells that carry the HLA-A, -B -C, and also the HLA-DR antigens identical to the original PBL

This work has been made possible by the repeated blood donation of Mrs Reefhuis and Mr van Delft The authors wish to thank Ms Janny Parlevliet for HLA-DR typing, Ms Corrie van Klink and Ms Lenneke Mulder of the Blood Transfusion Service of the Bloodbank of the University Medical Center in Leiden, Dr J D'Amaro and Dr M Giphart for reading, and Ms Anne Wies van Oosten and Ms C S L Mackenzie for editing, the manuscript

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