HELPER CELL-INDEPENDENT CYTOTOXIC CLONES IN MAN*

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Cloning technology has permitted the isolation and expansion of distinct T cell populations. Murine T helper (T_h) clones (cells that proliferate in response to alloantigen but are noncytotoxic) and T cytotoxic (T_c) clones (cells that are cytotoxic but fail to proliferate when cultured with antigen alone unless exogenous lymphokine is provided) have been reported, as has collaboration between them (1, 2). Recently, Widmer and Bach (3) described yet another murine T cell functional class, that of the “antigen-driven, helper cell-independent cytotoxic” (HITc) clone that mediates cytotoxicity and proliferates to alloantigen in the absence of exogenous lymphokine. In contrast to both T_h and T_c, whose existence and reactivities had been predicted from mixed lymphocyte culture (MLC) studies (4–6), it would have been extremely difficult to establish the existence of the HITc without cloning.

We have studied human T cell clones derived from two different sensitization procedures: (a) from a patient with malignant melanoma who had been allosensitized in vivo and whose cells had been restimulated in vitro with the patient’s autologous Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line (LCL), and (b) from a primary in vitro allosensitized MLC. Clones analogous to HITc were detected. A relative numerical predominance of HITc clones (14 out of a total of 19 clones studied) and the absence of classical T_h clones among those derived from the autologous LCL-primed culture contrasted sharply with the frequencies and variety of cell types derived from the in vitro allosensitized culture. In the latter situation, 22 of 38 clones were of the T_h class, whereas only 1 HITc clone was detected.

Materials and Methods

Primed Cultures. Peripheral blood lymphocytes (PBL) were isolated from healthy nontransfused donors A and L, and from a malignant melanoma patient, G, who had had four previous transfusions from different donors, each time with 200 cc leukocyte-rich concentrate prepared from 1 U whole blood. Primary MLC, using PBL from A (responder) and L (stimulator) were set up as previously described (7). Similarly, an autologous-LCL-primed culture was set up using in vivo allosensitized PBL from donor G (8). After 6 d culture, the primed populations were restimulated for an additional 6 d with stimulator cells in the presence of 20% human serum HS/RPMI containing 20% lectin-free T cell growth factor (TCGF). The latter was derived from sodium periodate-induced spleen-conditioned medium using a modification of the method previously described (9) and was used to induce clonal expansion of antigen-

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activated cells without causing lectin-induced activation of unprimed cells. The primed cells were then cloned by micromanipulation.

**Cloning by Micromanipulation.** Primed cells to be cloned were suspended in 5% HS/RPMI medium (200 cells/ml) and cell suspensions were pipetted into a 100-mm glass petri dish (Pyrex; Corning Glass Works, Science Products Div., Corning, NY). A single cell viewed under an inverted light microscope was isolated with a finely drawn sterile capillary pipette. The dish was then replaced by a second petri dish (serving as an intermediate chamber) containing 5 ml of medium alone. The contents of the pipette were discharged into the medium to ascertain that only a single cell had been picked up. The same cell was then deposited into individual microtiter U-bottomed wells (3799; Costar, Data Packaging, Cambridge, MA) containing 0.1 ml of PHA-induced TCGF (10). Irradiated stimulating cells (1 X 10^4/0.1 ml) were then added to each well. Subsequent clonal expansion was carried out in cluster wells (3524; Costar) and maintained as previously described (10).

**Antigen-induced Proliferative and Cell-mediated Lympholysis (CML) Assays.** Clonal populations were tested for proliferation using a standard primed lymphocyte typing (PLT) assay (11) and cytotoxicity assay (7) at predetermined effector/target (E/T) cell ratios. Before testing, cloned cells were washed once and left overnight in 15% HS/RPMI (2 X 10^6 cells/ml). Lectin-dependent cytolysis was performed by adding 0.05 ml of a 500/µg/ml stock of phytohemagglutinin-M (Difco Laboratories, Detroit, MI) to 0.1 ml test medium. Wells containing target cells alone were similarly treated.

### Results and Discussion

Multiple replicates of expanded clone cells were initially screened for cytotoxicity against specific (G-LCL) and autologous (G-PBL) target cells as previously described (7). The clones were similarly screened for antigen-induced proliferation against both G-LCL and G-PBL. A threefold or more increase in thymidine uptake after stimulation by G-LCL relative to that by G-PBL was regarded as positive.

Of 19 GG clones derived from G-LCL-primed cells tested in the screening assay, 14 showed positive responses for both proliferation and cytotoxicity, whereas 5 showed neither of the two reactivities (data not shown). Selected clones from these two classes of GG clones (seven from the former and two from the latter) were further tested for proliferative response in a PLT assay (11) and for cytotoxicity (see Materials and Methods). Proliferative reactions of four representative GG clones are presented in Table I. Clones GG-9, GG-16, and GG-2 proliferated when stimulated by the autologous G-LCL cells and by allogeneic JR-LCL cells; however, another allogeneic KZ-LCL stimulator failed to stimulate the same GG clones. These clones also failed to respond to autologous G-PBL, allogeneic LKC-PBL, or to a pool of PBL obtained from 10 unrelated healthy individuals. The antigen-driven GG clones were cytotoxic for those same cells (i.e., G-LCL and JR-LCL) that can stimulate them to proliferate but not for those that cannot (i.e., KZ-LCL) (Table II), including G-PBL (data not shown). Our data suggest a level of antigenic specificity with regard to both proliferation and cytotoxicity as expressed by these GG clones although the precise epitope(s) involved are currently not known.

Cloning by micromanipulation, using the intermediate chamber to deposit and again to draw up the same single cell, convinced us of the true clonality of the GG populations. Based on their dual capabilities to undergo antigen-induced proliferation as well as to mediate cytotoxicity, these GG clones were regarded as being analogous to the HITc clones first described in murine system (3). Three of the GG-HITc clones were characterized for cell surface determinants with OKT monoclonal antibodies and analyzed on a fluorescence-activated cell sorter (FACS IV; B-D FACS Systems,
### Table I

**Proliferative Response of GG Clones Derived from Autologous-LCL-primed Culture**

| Stimulator* cells | GG-9 | GG-16 | GG-2 | GG-8 | PLT cells‡ |
|------------------|------|-------|------|------|-----------|
| G-PBL±           | 0.4 ± 0.8 | 0.1 ± 0.3 | 0.2 ± 0.1 | 0.6 ± 0.0 |          |
| G-LCL±           | 1.9 ± 0.7 | 24.3 ± 0.9 | 27.0 ± 0.9 | 15.5 ± 0.9 | 1.6 ± 0.6 | 29.2 ± 1.1 |
| JR-LCL±          | 4.1 ± 0.1 | 30.6 ± 2.3 | 23.6 ± 1.2 | 13.9 ± 1.3 | 1.3 ± 0.9 | 15.2 ± 1.0 |
| KZ-LCL±          | 1.7 ± 0.1 | 2.4 ± 0.6 | 1.3 ± 0.8 | 2.2 ± 0.2 | 1.0 ± 0.1 | 14.5 ± 0.6 |
| LKC-PBL±         | 0.4 ± 0.2 | 0.8 ± 0.4 | 0.6 ± 0.3 | 0.5 ± 0.1 | 0.5 ± 0.0 | 17.3 ± 0.5 |
| Pool-PBL±        | 0.8 ± 0.6 | 1.6 ± 0.6 | 1.1 ± 0.2 | 0.5 ± 0.1 | 0.8 ± 0.2 | 13.0 ± 1.6 |

* HLA phenotypes: G (HLA-A2, A2, B7, Bw61, Cw1, DR7, MB2, MT3); JR (A2, B7, B37, DR2, MB1, MT1); KZ (A2, A11, B37, DR1, DR2, MB1, MT1); LKC (A2, A24, B60, Cw3, DR2, DR7, MB1, MB2).

‡ Day 10 cultured cells from healthy unrelated donor (R) primed to pool of lymphocytes from 10 unrelated individuals (12).

§ [¹H]thymidine incorporation in × 10³ cpm ± SD.

|| Pool of PBL from 10 unrelated individuals.

### Table II

**Cytotoxic Activity of GG Clones Derived from Autologous-LCL-primed Culture**

| Experiment* | Effectors clones | Target cells | Percent cytotoxicity |
|-------------|------------------|--------------|---------------------|
|             |                  |              | E/T ratio           |
|             |                  |              | 90:1 25:1 6:1 1:1 0.02:1 |
| 1           | GG-16 G-LCL      | NT‡ 52 ± 14§ | 27 ± 17 6 ± 5 NT    |
| 2           | GG-2 G-LCL       | NT 48 ± 12   | 36 ± 11 11 ± 6 NT   |
| 3           | GG-8 G-LCL       | NT 27 ± 7    | 23 ± 8 4 ± 3 NT     |
|             | GG-9 G-LCL       | 17 ± 2      | 15 ± 2 14 ± 2 13 ± 3 4 ± 4 |
|             | JR-LCL           | 18 ± 2      | 22 ± 2 20 ± 2 13 ± 3 6 ± 5 |
|             | KZ-LCL‡          | 1 ± 1       | 1 ± 1 -1 ± 2 -1 ± 2 -7 ± 1 |

* Data presented in each experiment were representative of experiments carried out on two separate occasions. Spontaneous release of target cells in experiment 1 was 20%; in experiment 2 was 14%; experiment 3 were: G-LCL (25%); JR-LCL (24%); KZ-LCL (48%).

† Not tested.

§ Percent lysis expressed as ([test pm - spontaneous pm] + [maximum pm - spontaneous pm]) X 100.

In the same experiment, KZ-LCL target cells were lysed by control effectors (day 6 cultured PBL sensitized to a pool of lymphocytes (13).

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Becton, Dickinson & Co., Sunnyvale, CA). All were found to be T3⁺T4⁻T8⁺ (data not shown). Clone GG-8 represents the class of clones (5 of 19) that showed neither cytotoxicity nor proliferation to the sensitizing cell (G-LCL) but can be maintained in TCGF.

Using the same cloning technique, human clones were derived from primary in vitro allosensitized cultures (designated as AL clones). Of 38 AL clones tested in screening assays, 22 (group A) responded proliferatively but showed no cytolsis; 2 (group B) showed cytolsis but failed to proliferate; 1 (group C) showed both proliferation and cytolsis; and 13 (group D) showed neither of the two reactivities (data not shown). Three clones from group A, four from group D, and all of the clones of groups B and C were further tested at known cell concentrations for both reactivities (see Materials and Methods). Proliferative and cytotoxic results from four AL clones, representing the four different functional subsets defined above, are presented in Table III. The subsets are: (a) clone AL-26, representing the Th class, which responds proliferatively to the sensitizing alloantigen and is noncytotoxic. 3 of these 22 clones (including AL-26) were further tested and found to secrete TCGF-like
lymphokine upon antigenic stimulation (Wee et al., manuscript in preparation) and
to express cytotoxicity in the presence of phytohemagglutinin (see Legend to Table
III); (b) a class of conventional helper-dependent cytotoxic Tc clone, cells of which
proliferate in the presence of TCGF, represented by clone AL-77; (c) a single clone
(AL-23), analogous to the HITc clones; (d) a class of 13 AL-clones, represented by
clone AL-7, exhibiting none of the above reactions; four of these clones were tested
and lysed NK-sensitive K562 targets (data not shown). When selected AL clones were
characterized for cell surface determinants, only the natural killer-like clones (four
tested) were T3+T4−T8+ whereas all other clones tested (3Th, 2Tc, and 1 HITc) were
T3+T4+T8+ (data not shown).

Based on previous findings (7) and our data here, positive responses (both prolif-
erative and cytotoxic) detected in screening assays have been found to be consistently
predictive of positive responses that were confirmed by subsequent standard PLT and
CML assays. “False negatives,” on the other hand, could occur due to insufficient
numbers of effector or responding cells used in screening assay. Our data, however,
would argue that if such false negatives occur at all, they are relatively infrequent. Of
a total of 10 clones that were negative in the initial screening cytotoxicity assay (2
from the GG group and 8 from the AL group), all remained negative when tested at
known E/T ratios. Similarly, all eight nonproliferative clones in the screen (six AL
clones and two GG clones) remained nonreactive when tested in a standard PLT
assay.

The repertoire of cell types derived from allosensitized cultures was evidently
different from that derived from the autologous-LCL-primed cultures. The relative
rarity of HITc (1 out of 38 clones tested) among the allosensitized AL clones was in
sharp contrast to their predominance (14 out of 19 clones tested) among the GG clones
derived from autologous-LCL-primed cultures. Furthermore, the absence of Th clones

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**Table III**

| Stimulators | TCGF‡ | AL-26 | AL-77 | AL-23 | AL-7 |
|-------------|-------|-------|-------|-------|------|
| Ax          | −     | 0.6 ± 0.0§ | 0.3 ± 0.0 | 0.4 ± 0.0 | 0.3 ± 0.0 |
|             | +     | 18.5 ± 2.4 | 6.7 ± 0.9 | 8.2 ± 0.5 | 12.9 ± 4.3 |
| Lx          | −     | 4.7 ± 1.1 | 0.3 ± 0.0 | 4.1 ± 0.5 | 0.2 ± 0.0 |
|             | +     | 19.3 ± 1.3 | 9.5 ± 0.3 | 13.5 ± 0.2 | 16.2 ± 0.7 |
|             | --    | NT† | 3.2 ± 0.3 | NT | 13.8 ± 0.3 |

Percent cytotoxicity

| E/T: ND‡ | 2 ± 4 | 21 ± 4 | 33 ± 2 | −7 ± 6 |
| E/T: 50:1 | −5 ± 2** | 20 ± 5 | 17 ± 2 | 3 ± 2** |

* HLA phenotypes: donor A (A1, A2, Bw6, Cw-, DR3, DR5); donor L (A1, A2, Bw6, Cw-, DR2, DR7).
‡ PHA-conditioned medium (TCGF) was added to test cultures at a final concentration
of 30%.
§ [3H]thymidine incorporation in (× 10⁻⁶ cpm) ± SD.
† Not tested.
¶ E/T not determined in screening assay (7).
** When tested in the presence of lectin (see Materials and Methods) at three E/T ratios
starting with 50:1, AL-26 showed 22.3 ± 3, 18.7 ± 9, 10.1 ± 4, and AL-7 showed 25
± 2, 16 ± 5, 13 ± 1. Spontaneous release of target cells (phytohemagglutinin blasts)
was 26%.
in the 19 clones derived from autologous-LCL primed cultures is highly significant, considering the frequency of Th (22 out of 38 AL clones tested) clones found in allosensitized culture.

This difference in the frequencies of cell types may be related to one or more of the following. First, the disease state of donor G might have a significant bearing on the results obtained, although indirect evidence suggests that this is unlikely, in that the OKT phenotypes of blasts obtained from donor G at various times following allo- or autologous-LCL in vitro stimulations are similar to those from healthy donors. For example, after 6 d primary allosensitizations, cells from donor G and healthy donors expressed similar T4/T8 ratios (2:1–3:1). In contrast, after autologous-LCL sensitizations, cells from both G and healthy donors have lower T4/T8 ratios (0.4:1–1:1) (8; unpublished data). Thus, this suggests that different sensitization procedures may exert a greater influence on the types of response observed than does the health of the individuals. Second, HITc may preferentially respond to antigens of modified autologous cells, e.g., an LCL, or to non-major histocompatibility complex (MHC) alloantigens rather than to MHC antigens. The apparent absence of Th among clones derived from autologous-LCL-primed culture may be interpreted as further indications that either the precursor populations responsive to alloantigens are different from those activated by modified self antigens or that such cells do not predominate in "memory" responses (14). Third, HITc may arise from memory T populations present in donor GG, who has not only been in vivo alloimmunized, but is also EBV positive. Studies in mouse suggest a similar possibility based on isolations of HITc reactive to minor histocompatibility antigens1 and to K region differences only.2 Notably, in both these cases, as in ours, clones were derived after in vitro restimulation of in vivo sensitized cells.

The fact that HITc, unlike conventional Tc, can autonomously mediate cytolysis and, as shown here, can be directed against autologous-LCL cells (serving as an in vitro model of an abnormal cell such as a tumor) raises the possibility of investigating the potential application of such cell types for tumor immunotherapy. Such investigations are particularly pertinent in view of recent findings in mouse (15) demonstrating that HITc, rather than the conventional Tc, can eliminate allogeneic tumor in vivo.

Summary

We report here a class of helper cell-independent cytotoxic T cell (HITc) clones in man that can proliferate in response to antigenic stimulation as well as mediate cytocytotoxicity. HITc appear to be rare among clones derived from primary in vitro allosensitized culture, but constitute the majority of clones derived from cells sensitized to autologous Epstein-Barr virus-transformed lymphoblastoid cell lines. The implications of the derivation and function of HITc clones are discussed.

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