A CLONED HUMAN T CELL LINE CYTOTOXIC FOR AUTOLOGOUS AND ALLOGENEIC B LYMPHOMA CELLS

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Activation of peripheral blood cells (PBL)1 of cancer patients with autologous tumor cells (1) or in conventional mixed lymphocyte culture (MLC) with allogeneic PBL of a single donor (2, 3), a pool of 10–20 unrelated individuals (3, 4) or with an Epstein-Barr Virus (EBV) transformed lymphoblastoid B cell line (5) has been shown to result in the induction of cytotoxicity against autologous tumor cells. These studies have indicated that the PBL of cancer patients contain precursors of cells cytotoxic for autologous tumor cells. However, the heterogeneity of bulk T cell cultures, especially of those activated in MLC, prevents a meaningful analysis of the effector- and target cell antigens involved in autotumor cell cytotoxicity. Recently we demonstrated that cloned lines of cytotoxic T lymphocytes (CTL) that lysed autologous, short-term cultured melanoma cells, could be established in MLC and mixed lymphocyte tumor cell culture (MLTC) (5). Both T4+ and T8+ CTL clones were isolated and were shown to preferentially lyse melanoma cells. This cytotoxicity was not HLA-restricted. The reactivity of the CTL clones was found to differ from that of CTL clones specific for HLA antigens, since the reactivity was not blocked with monoclonal antibodies against HLA antigens. Moreover, antibodies against T3, T4, and T8 that block the activity of T4+ and T8+ CTL clones against class II and class I MHC antigens (6, 7), were not able to inhibit the auto-tumor cell reactivity. Similar CTL clones with a restricted cytotoxic pattern lysing only autologous melanoma cells have been described (8).

In our previous studies, short-term cultured melanoma cells were used as target cells. Tissue culture of tumor cells may result in an increased susceptibility to nonspecific cytotoxic effects. We could, however demonstrate that the melanoma target cells used were not susceptible to T cell bulk cultures having a high activated killer (AK) cell activity against the myeloid/erythroid human natural killer (NK)- and AK-sensitive target cell K562. However, to avoid any interferences of tissue culture of the target cells we investigated whether similar CTL clones could also be generated against fresh, noncultured autologous human H. Yssel is the recipient of a fellowship from the “Koningin Wilhelmina Fonds” (Netherlands Cancer Foundation). Correspondence and reprint requests should be addressed to Dr. Yssel.

1 Abbreviations used in this paper: AK, activated killer; Con A, concanavalin A; CTL, cytotoxic T lymphocyte; EBV, Epstein-Barr virus; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; HLA, human major histocompatibility gene complex; IL-2, interleukin 2; LFA-1, leukocyte function-associated antigen; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; MLTC, mixed lymphocyte tumor cell culture; MPL, autologous B lymphoma cells; NK, natural killer; PBL, peripheral mononuclear blood lymphocytes.

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lymphoma cells. In this paper it is demonstrated that cloned CTL lines with strong cytotoxic activity against autologous fresh lymphoma cells could be isolated from PBL, obtained from a B cell lymphoma patient, and activated by cocultivation with autologous lymphoma cells. Analysis of the specificity of the CTL clones obtained, indicated that in addition to the autologous lymphoma cells, allogeneic fresh lymphoma target cells were also lysed, whereas most other target cells of different origin were not killed. Furthermore it is demonstrated that LFA-1, but neither T3 nor T4, plays a role in the activity of the B lymphoma specific CTL clones.

Materials and Methods

**Media and Reagents.** All experiments and cell cultures were carried out in serum-free medium as described (9). The composition of serum-free medium was based on Iscove's modification of Dulbecco's modified Eagle's medium, referred to as Iscove's, and was reconstituted according to the manufacturer's directions (Gibco Biocult, Glasgow, Scotland) with distilled, deionized water to which 3.024 g/l NaHCO₃ (Merck, Darmstadt, W. Germany) was added. The medium was supplemented with ethanolamine (2 × 10⁻⁵ M, Merck), bovine serum albumin (BSA, 0.25%), transferrin (35 µg/ml), insulin (5 µg/ml), linoleic acid, oleic acid, and palmitic acid (1 µg/ml), purchased from Sigma Chemical Co., St. Louis, MO. Penicillin and streptomycin (Gibco) were added to a final concentration of 100 U/ml). Stock solutions of transferrin (20 mg/ml) and insulin (2.5 mg/ml) were prepared in phosphate-buffered saline (PBS) and 0.01 N HCl, respectively. The saturated and unsaturated fatty acids were first dissolved in ethanol (Merck) (5 mg/ml) and then diluted to the appropriate concentration in Iscove's.

**B Lymphoma Cells.** The autologous B lymphoma cells that were used as stimulator and target cells in this study were obtained from pleural effusions and referred to as MPL. The other allogeneic B lymphoma cells were collected from spleen extirpations of B lymphoma patients.

Viable tumor cells were separated from nonviable cells and erythrocytes by centrifugation (15 min, 800 g) over Ficoll/Hypaque. After two washings at 4°C with PBS supplemented with 0.2% BSA, the cells were resuspended in RPMI-1640 (Grand Island Biological Co., Grand Island, NY), containing 1% BSA, and an equal volume of ice-cold RPMI-1640 containing 1% BSA and 20% dimethyl sulfoxide (DMSO) was added dropwise over a period of 3–5 min. The cells were frozen in a cooling chamber according to a temperature-controlled program and stored in liquid nitrogen. After thawing, the cells were used as target cells for the CTL clones. If necessary, dead cells were removed by centrifugation over Ficoll/Hypaque.

**Description of the B Lymphomas.** All the B lymphomas used in this study were of advanced stage IV. The further characteristics of the B lymphomas were as follows:

- **MPL:** centrocytic/centroblastic, poorly differentiated, diffuse; surface markers IgM, IgD, kappa. **LIV:** centrocytic/centroblastic, well-differentiated, diffuse; surface markers IgM, kappa. **KOS:** centrocytic, well-differentiated, diffuse; surface markers IgM, IgD, kappa. **TOP:** centrocytic, poorly differentiated, diffuse; surface markers IgM, IgD, IgG, kappa. **STS:** centrocytic/centroblastic, poorly differentiated, diffuse; surface markers IgM, IgD, kappa. **KOR:** centrocytic/centroblastic, poorly differentiated, diffuse; surface markers IgG, lambda. **PLN:** centrocytic/centroblastic, poorly differentiated, diffuse; surface markers IgM, IgD, IgG, lambda. **POS:** centrocytic/centroblastic, poorly differentiated, nodular; surface markers IgM, lambda.

- **PBL.** Mononuclear leukocytes from a 66-year old female patient with centrocytic/centroblastic B cell lymphoma were obtained by centrifugation over a Ficoll/Hypaque mixture and were enriched for T cells by rosetting with sheep erythrocyte SE as described previously (9). The HLA phenotype of this patient was HLA-A1,2;B8,w62;Cw3;DRw3,6.

**Mixed Leukocyte Tumor Cell Culture (MLTC).** MLTC was carried out in Iscove's medium in 2-ml tissue culture plates (Linbro, no. 74-033-05, Linbro, Hamden, CT). 10⁶ responder
T cells were stimulated with 10⁶ irradiated (3,000 rad) MPL. On day 10 secondary MLC was carried out, in which the remaining PBL were restimulated with irradiated MPL. 4 d later the PBL were collected, washed with serum-free medium, and cloned by limiting dilution.

Cloning by Limiting Dilution. Cloning by limiting dilution was carried out in serum-free medium as described previously (9). After secondary stimulation the PBL were collected, washed, and seeded in flat-bottomed microtiter wells (Costar, no. 3596) at a concentration of 1 cell/well in the presence of a feeder mixture consisting of 10⁶ allogeneic PBL, 10⁴ autologous lymphoma cells (both irradiated, 3,000 rad), and 0.1 μg/ml PHA or 2% (vol/vol) of an IL-2-containing supernatant (9) in a final volume of 0.1 ml. After 10–14 d culturing in a humidified atmosphere of 5% CO₂ at 37°C proliferating clones were transferred to 1-ml wells (Linbro, no. 74-033-05) and weekly supplied with the feeder cell mixture described above.

Monoclonal Antibodies. Monoclonal antibody OKT4A, which reacts with a subset of T cells including the helper/inducer T cells (10), was obtained from Ortho Pharmaceutical Corp., Raritan, NJ. The SPV monoclonal antibodies used in this study were produced by hybridomas that were obtained by fusing SP 2/0 myeloma cells (11) with spleen cells from mice immunized with the T4+ clone HG-38 or with the T8+ clone HC-31, and were selected according to their capacity to inhibit the cytotoxic reactivity of these clones (7, 12). SPV-T3a, SPV-T3b, SPV-T3c, and SPV-T8 have all the characteristics of antibody specific for the T3 molecular complex (OKT3, Leu-4) (13, 14) and T8 (OKT8, Leu-2a) (15, 16), respectively. The monoclonal antibodies SPV-L1 and SPV-L5 inhibited the cytotoxic reaction of T8+ and T4+ CTL clones specific for class I and class II MHC products. They precipitated a complex of two polypeptides with a mol wt of 95,000 and 160,000, suggesting that SPV-L1 and SPV-L5 recognize a human leukocyte function-associated antigen (LFA-1) (17). The monoclonal antibody SPV-L3 precipitated a bi-molecular complex with a mol wt of 28,000 and 34,000 and was shown to recognize a determinant on HLA-DC (12). The monoclonal antibody Mas 015 (W6/32) recognizing a monomorphic determinant on HLA-A, B, and C antigens (18) was purchased from Sera Lab Ltd., Sussex, England. The monoclonal antibody Q 5/13, recognizing a monomorphic determinant on the HLA-DR chain (19), was kindly provided by Dr. S. Ferrone from the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York.

Assays for Determination of Cytotoxic Activity. Cytotoxic activity of the CTL was measured in a ⁵¹Cr release assay, which was carried out in round-bottomed microtiter plates (Linbro, Titertek) in a volume of 200 μl. 10⁴ ⁵¹Cr-labeled lymphoma target cells (or 10⁵ lymphoid nonlymphoma cells) were incubated with various numbers of CTL effector cells in Iscove’s medium supplemented with 0.25% BSA. The plates were centrifuged for 5 min at 50 g and incubated during 4–8 h at 37°C in an atmosphere of 5% CO₂. Then 100 μl of supernatant were collected from each well and counted in a gamma counter (Beckman). All tests were carried out in triplicate. The percentage-specific ⁵¹Cr release was calculated according to the formula:

\[ \text{Release in sample} - \text{Release in medium} / \text{Release in Triton X-100} \times 100\% \]

Inhibition of Cytotoxic Activity of the CTL Clones with Monoclonal Antibodies. Inhibition of cytotoxicity with monoclonal antibodies was determined in a cytotoxic inhibition assay. 10⁵ ⁵¹Cr-labeled target cells were incubated in the absence or presence of 10 μl appropriately diluted monoclonal antibody for 20 min at room temperature. Then 20 μl effector cells were added. The mixture was centrifuged at 50 g during 5 min and incubated as described above. After the incubation period 150 μl ice-cold medium were added. The plates were centrifuged at 50 g for 5 min after which 100 μl was harvested and counted. The percentage inhibition was calculated as follows:

100 − % Lysis in the presence of monoclonal antibody / % Lysis in the absence of monoclonal antibody × 100%.
**Phenotyping with Monoclonal Antibodies.** Phenotyping of the CTL clones was carried out by indirect immunofluorescence as follows: $5 \times 10^5$ CTL were washed with PBS supplemented with 0.2% BSA and 0.1% NaN₃ and incubated with 40 µl of an appropriate dilution of the monoclonal antibodies OKT4 (Ortho) or SPV-T8 for 30 min at 4°C. The cells were washed three times in a refrigerated centrifuge and incubated with FITC-labeled goat anti-mouse Ig (Nordic, Tilburg, The Netherlands) for 30 min at 4°C. Finally the cells were washed three times and analyzed using a FACS IV.

**Modulation of the T3 Determinant.** Antigen modulation of the T3 complex from the CTL clones was induced by incubation with anti-SPV-T3b monoclonal antibody for 24 h at 37°C. The CTL cells were cultured in Iscove's at a concentration of $4 \times 10^6$ cells/ml with a final dilution of anti-SPV-T3b monoclonal antibody of 1:100 in the presence of 2 $\times 10^5$/ml MPL cells. CTL clones, incubated with MPL cells only, served as negative control. After modulation the cells were centrifuged over Ficoll/Hypaque to remove dead cells, collected, and washed extensively. Control and SPV-T3b modulated cells were analyzed for reactivity with anti-SPV-T3b monoclonal antibody by measuring indirect fluorescence on a FACS IV cell sorter using FITC-conjugated goat anti-mouse Ig (Nordic, Tilburg, The Netherlands). Untreated cells incubated with anti-SPV-T3b and PBS served as positive and negative control, respectively.

**Results**

**Generation of Cytotoxic Activity in Primary and Secondary MLTC.** PBL from a B cell lymphoma patient were enriched for T cells by rosetting with SRBC and the E+ cells were stimulated in primary MLTC and after another 10 d in secondary MLTC by cocultivation with MPL cells. As is shown in Table 1, the primary stimulated T cells were not cytotoxic for the MPL cells, but restimulation resulted in the appearance of CTL activity indicating that the culture contained clones cytotoxic for MPL cells.

**Isolation and Screening of Cytotoxic Clones with Auto-tumor Reactivity.** 7 d after secondary stimulation the remaining cells were restimulated in the presence of allogeneic PBL, MPL cells, and PHA and were cloned 7 d later by limiting dilution. In only 6/10 wells in which 1,000 cells/well were seeded, growing cells

| Effector cells | Percentage of specific lysis |
|---------------|-----------------------------|
|               | Effector/target cell ratio  |
|               | 5:1 | 20:1 | 40:1 |
| A: Primary MLTC* | 2 | 2 | 3 |
| B: Secondary MLTC* | 12 | 20 | 42 |
| C: Cloning of B on 1,000 cells/well | 25 | 41 | 63 |
| D: Cloning of C on 0.5 cells/well | 30 | 41 | 44 |
| MWS-14 | 32 | 45 | 53 |

* Effector cells were E+ cells tested on day 7 after primary MLTC.
* Tested on day 4 after onset secondary MLTC.
* Subcloning of MWS-14 yielded 34 subclones with comparable lytic activity against autologous MPL cells.
were observed. Cells from one of these six wells were shown to be cytotoxic for the MPL cells. These cytotoxic cells were seeded at a concentration of ~0.5 cells/well in the presence of the feeder cell mixture. Only 4/10 wells contained proliferating cells. From these four wells one clone (MWS-14) showed a stable lytic activity against the autologous target cells.

Phenotyping revealed that clone MWS-14 expressed the T3+, T4+, and T8- surface markers. To exclude the possibility that this clone was a mixture of two clones, it was subcloned at a concentration of ~0.5 cells/well and two subclones were selected for further investigation.

Specificity of the CTL Clones. The specificity of the CTL clone MWS-14 and the subclones MWS-14-30 and MWS-14-34 was determined by testing the cytotoxic activity against a panel of cells originating from various normal tumor tissues, including a large number of target cells which are known to be very susceptible for NK-cell killing. Table II shows that seven of seven B lymphoma cell types were lysed by the CTL clones, indicating that all B lymphoma cell types tested express determinants recognized by MWS-14. In contrast to the general activity against B lymphoma cells, MWS-14-30 and MWS-14-34 were cytotoxic for only 3 of 23 target cells, respectively 2 of 23 normal- and malignant lymphoid and nonlymphoid target cells (Table III). Importantly, the two subclones failed to lyse an EBV-transformed B cell line derived from PBL of one of the B lymphoma patients (KOS) whose B lymphoma cells were killed (Table II). The EBV-transformed B cell line KOS had the same HLA phenotype as the KOS B lymphoma cells, but did not react with a monoclonal antibody directed against the idioype of the Ig present on KOS B lymphoma cells (E. Rankin and A. Hekman, submitted for publication). In addition the subclones failed to lyse PHA-activated PBL from any of the B lymphoma patients (Table III). It is of interest to note that although both subclones, MWS-14-30 and MWS-14-34, killed all lymphoma cells to the same extent, the subclones differed in their killing activity against HSB and T24. The reason for this difference is unknown, but it may be due to the fact that in some instances CTL clones may acquire nonspecific cytotoxic activities that can be measured against particular sensitive target cells, as has been described for allospecific mouse CTL clones (20). Taken together the data presented in Tables II and III indicate that the CTL clones MWS-14-30 and MWS-14-34 recognize antigens preferentially expressed on malignant B lymphoma cells.

Fresh MPL Cells Are Resistant to Lysis by Allogeneic PBL Containing NK/AK Cells and by CTL Clones Directed at HLA Specificities Not Present on the MPL Cells. It has been described that lysis of human tumor cells in T cell-mediated cytotoxicity can be contributed to "natural killer" (NK) or "activated killer" (AK) activity in which specific antigen recognition is not involved (21, 22). This type of cytotoxic reaction, mediated by activated T cells, is defined by the lysis of the human erythroid/myeloid cell line K562 (23, 24). PBL stimulated with PHA or allogeneic EBV-transformed B cells are highly cytotoxic for K562 (reference 25; Table IV) but it is also shown that such activated T cells fail to lyse the MPL cells. It is furthermore shown in Table IV that a cloned T cell line (POS-41) isolated from PBL stimulated with PHA, which was highly cytotoxic for K562, was not cytotoxic for MPL cells. The MPL cells express HLA-A2 and HLA-DC-1 and as
TABLE II

Cytotoxic Pattern of Clone MWS-14 and Subclones MWS-14-30 and MWS-14-34 Tested Against a Panel of Human Normal and Nonlymphoma Cells

| Target cells          | Percentage of specific $^{31}$Cr-release* CTL clone* |
|-----------------------|-------------------------------------------------------|
|                       | MWS-14 | MWS-14-30 | MWS-14-34 |
| HL-60 (promyelocytic leukemia) | 3      | 2         | 2         |
| U937 (histiocytic leukemia)   | 5      | 6         | 7         |
| K562 (erythroid/myeloid leukemia) | 4    | 9         | 9         |
| KG-1 (myelocytic leukemia)   | 0      | 1         | 2         |
| DAUDI (Burkitt's lymphoma)   | 6      | 8         | 8         |
| HPB-ALL (T leukemia)         | 21     | 31        | 24        |
| CEM (T leukemia)             | 18     | 16        | 26        |
| JV (EBV-transformed B cell line) | 7    | 2         | 2         |
| KOS-EBV (EBV-transformed B cell line) | 10  | 12        | 12        |
| CRIS (EBV-transformed B cell line)  | 14  | 12        | 11        |
| HHK (EBV-transformed B cell line)  | 8     | 7         | 0         |
| APD (EBV-transformed B cell line)  | 4     | 6         | 2         |
| AVL (EBV-transformed B cell line)  | 2     | 1         | 1         |
| LAZ 475 (EBV-transformed B cell line)  | 3    | 1         | 1         |
| E-14-17 (EBV-transformed B cell line)  | 6    | 7         | 1         |
| A 549 Cl 8 (broncho alveolar carcinoma) | 2    | 1         | 1         |
| MCF-7 (mamma carcinoma)      | 5      | 5         | 4         |
| CAMA (mamma carcinoma)       | 4      | 3         | 1         |
| T24 (bladder carcinoma)      | 25     | 28        | 16        |
| MEWO (melanoma)             | 2      | 1         | 1         |
| KER (melanoma)              | 7      | 11        | 6         |
| BUR (melanoma)              | 2      | 0         | 1         |
| BAT (fibroblast)             | 1      | 0         | 0         |
| MPL (autologous B lymphoma as control) | 42  | 45        | 48        |

* Cytotoxicity was also measured at E/T ratios of 10:1 and 20:1. The reaction pattern was the same as the one obtained at E/T ratio of 5:1. % specific $^{31}$Cr-release <15% at any E/T ratio were considered as negative, since the average percentage of lysis obtained with different (control) CTL clones, augmented with two times the SD never exceeded 15% lysis.

1 E/T ratio 5:1.

KOS-EBV is the EBV-transformed B cell line from B lymphoma patient KOS (see Table III).

expected, they were killed by the T8+ HLA-A2 specific CTL clone JR-2-16 and the T4+ HLA-DC-1 specific CTL clone JR-2-19 (6, 7).

In contrast, CTL clones directed at HLA determinants not present on the lymphoma cells (HG-31, LIV-8, directed against HLA-B7, and HG-38, directed against an unknown HLA-DC product) failed to kill both MPL cells and K562 cells. Since the MPL cells are not susceptible to nonspecific lysis and were only lysed by allospecific CTL clones recognizing MHC determinants present on the lymphoma cells, it is concluded that the anti-lymphoma activity of the CTL clone MWS-14 is antigen specific.

MHC Antigens Are Not Involved in the Cytotoxic Reactivity of the CTL Clones Against the MPL Cells. The activity of the CTL clones against allogeneic lymphoma cells already indicated that the activity of clone MWS-14 is not restricted by HLA products. It cannot be excluded, however, that clone MWS-14 recog-
Table III
Cytotoxic Pattern of CTL Clone MWS-14 and Subclones MWS-14-30 and MWS-14-34 Tested Against a Panel of Human Lymphoma Cells and Normal T Cells from the Same Donors

| Target cells*       | HLA phenotype | Percentage of specific 51Cr release CTL clone† |
|---------------------|---------------|----------------------------------------------|
|                     | A  | B  | C  | DR | MWS-14 | MWS-14-30 | MWS-14-34 |
| MPL lymphoma        | 1,2| 8, w62| w3, w7| w3, w6| 46 | 46 | 41 |
| T cells             | 3  | 3  | 5  |     |     |     |     |
| LIV lymphoma        | 2, w31| w44, w60| w3  | w2, —| 44 | 49 | 37 |
| T cells             | 7  | 4  | 5  |     |     |     |     |
| KOS lymphoma        | 1,11| 12, 17| w5, w7| w2, w4| 45 | 48 | 53 |
| T cells             | 8  | 4  | 5  |     |     |     |     |
| TOP lymphoma        | 3, w33| 17, w35| w3, w6| w1, w6| 30 | 24 | 31 |
| T cells             | 8  | 5  | 10 |     |     |     |     |
| STS lymphoma        | 2, w35| 13, w35| w4  | w2, w7| 36 | 38 | 40 |
| T cells             | 2  | 1  | 5  |     |     |     |     |
| KOR lymphoma        | 2, w24| w44, w38| — | w2, w7| 27 | 25 | 29 |
| T cells             | 6  | 6  | 9  |     |     |     |     |
| PLN lymphoma        | NT |     |     |     | 42 | 46 | 48 |
| T cells             | 8  | 3  | 4  |     |     |     |     |
| POS lymphoma        | NT |     |     |     | 39 | 42 | 36 |
| T cells             | 2  | 2  | 6  |     |     |     |     |

* As T cell were used PHA blasts of E+ cells.
† E/T cell ratio 10:1.
‡ Not tested.

nizes putative "alien" HLA antigens, which may be expressed on malignant B lymphoma cells. To further investigate the involvement of HLA antigens in the killing activity of MWS-14 against MPL cells, blocking studies were carried out with anti-class I MHC and anti-class II MHC antibodies which were shown previously to block very efficiently the activity of allospecific CTL clones (6, 7). Table V shows that the activity of JR-2-16 (HLA-A2 specific) against MPL was also blocked effectively by W6/32 (18). Furthermore, the lysis of MPL by the CTL clones JR-2-10 directed at HLA-DRw6, and JR-2-19, directed at HLA-DC-1, was blocked by the monomorphic antibodies Q 5/13 (anti–HLA-DR) (19) and SPV-L3 (anti-HLA-DC/DS) (12), respectively. These four antibodies were all found to react with the MPL cells (not shown). In contrast, the activity of MWS-14 and MWS-14-34 against MPL was not blocked by any of the anti-class I MHC or anti-class II MHC specific antibodies. The monoclonal anti-class I and anti-class II MHC antibodies also failed to block the activity of MWS-14 against allogeneic B lymphoma cells (not shown). Taken together these data indicate that the cytotoxic activity of MWS-14 against autologous and allogeneic B lymphoma cells is not directed against "alien" HLA antigens, nor MHC restricted.

T Cell Differentiation Antigens Are Not Involved in the Cytotoxic Reaction of the CTL Clones. Blocking studies with monoclonal antibodies directed against T3, T4, and T8 have shown that these antigens are involved in the cytotoxic activity of T4+ CTL clones directed at class II (26, 27) and of T8+ CTL clones directed...
### Table IV

*Fresh MPL Cells Are Resistant to Lysis by Allogeneic PBL Containing NK/AK Cells and by CTL Clones Directed at HLA Specificities Not Present on MPL Cells*

| Effector cells* | Percentage of specific $^{51}$Cr release target cells |
|-----------------|-----------------------------------------------------|
|                 | MPL  | K562 |
| Bulk T cell (MLC)$^\dagger$ |     |      |
| E/T cell ratio  | 40:1 | 11   | 92  |
|                 | 20:1 | 9    | 85  |
|                 | 10:1 | 7    | 86  |
|                 | 5:1  | 4    | 83  |
| Bulk T cell (PHA)$^\ddagger$ |     |      |
| E/T cell ratio  | 40:1 | 12   | 53  |
|                 | 20:1 | 7    | 35  |
|                 | 10:1 | 2    | 19  |
|                 | 5:1  | 3    | 10  |
| POS-41          |      | 7    | 49  |
| JR-2-16         |      | 59   | 6   |
| JR-2-19         |      | 25   | 7   |
| HG-31           |      | 3    | 6   |
| LIV-8           |      | 6    | 2   |
| HG-38           |      | 8    | 3   |
| MWS-14          |      | 44   | 4   |
| MWS-14-34       |      | 47   | 9   |

* E/T cell ratio of the CTL clones 10:1.
$^\dagger$ Tested 7 d after onset MLC.
$^\ddagger$ Tested 4 d after PHA stimulation.

### Table V

*MHC Antigens Are Not Involved in the Cytotoxic Reaction of CTL Clone MWS-14 Against MPL Cells and Allogeneic Lymphoma Cells*

| CTL clone* | Target cell | Lysis | Percentage of specific inhibition in the presence of$^\ddagger$ |
|------------|-------------|-------|-------------------------------------------------------------|
|            |             |       | W6/32 Q 5/13 SPV-L3                                         |
| JR-2-16    | MPL         | 53    | 80 1 3                                                      |
| JR-2-10    | MPL         | 32    | 0   50 0                                                    |
| JR-2-19    | MPL         | 25    | 0   90                                                      |
| JR-2-19    | KOS         | 30    | 0   70                                                      |
| JR-2-19    | POS         | 30    | 10  3 10                                                   |
| JR-2-19    | LIV         | 34    | 0   1 0                                                    |
| JR-2-19    | KOS         | 38    | 1   0 0                                                    |

* E/T ratio 10:1.
$^\ddagger$ Dilution of 1:100 ascites fluid.
at class I MHC antigens (6, 7). As expected, OKT4A and SPV-T8 are able to block the activity of T4+ CTL clone JR-2-19 and the T8+ clone JR-2-16 against the MPL cells (Table VI). In addition anti-T3 reagents (SPV-T3a, SPV-T3b) were able to block efficiently the cytotoxic activity of JR-2-16 and JR-2-19 (Table VI). In contrast, none of the anti-T4 or anti-T3 reagents inhibited the activity of the T3+ T4+ clone MWS-14 against the MPL.

Anti-T3, however, not only blocks but also induces cytotoxicity and therefore blocking of the cytotoxic reaction can be masked by a high induction of cytotoxicity (H. Spits, manuscript submitted for publication). Therefore we decided to modulate the T3 complex from the effector cells with anti-T3 antibody (28). Fig. 1a illustrates that after incubation of clones MWS-14 and JR-2-19 with SPV-T3b, >90% of the T3 was removed and the cytotoxic activity of the modulated JR-2-19 against the MPL cells was abolished (Fig. 1b). Addition of 10 μg/ml Con A restored the cytotoxic activity, indicating that the loss of function was not due to an aspecific inactivation of the CTL clone. In contrast, the cytotoxic activity of clone MWS-14 against the autologous lymphoma cells was not affected by modulation of the T3 complex (Fig. 1b). Furthermore, addition of Con A did not enhance the cytotoxic activity of this CTL clone. These results confirmed the inhibition experiments with anti-T3 and indicated that T3 is not associated with the cytotoxicity mediated by MWS-14.

Human LFA Is Involved in the Cytotoxic Reaction of MWS-14. Monoclonal antibodies directed at the function-associated antigen LFA-1 block antigen-specific CTL-mediated killing (12, 17, 29) as well as NK (12, 29), AK (12), K cell activity (F. Miedema et al., submitted for publication) and lectin-dependent cytotoxicity (12). Table V demonstrates that two monoclonal antibodies, SPV-L1 and SPV-L5, which recognize LFA-1 (12), block the activity of MWS-14 against MPL cells, indicating that LFA-1 is involved in this cytotoxic reaction.

Discussion

In this paper a novel type of T3-independent, antigen-specific anti-lymphoma cytotoxicity mediated by a T3+ T4+ T8- CTL clone directed against fresh

### Table VI

| CTL clone* | Lysis | Percentage of specific inhibition in the presence of ** | ** |
|---|---|---|---|
| | | SPV-T3b OKT4A SPV-T8 SPV-L1 SPV-L5 |
| JR-2-16 | 40 | 37 3 51 | 65 55 |
| JR-2-10 | 45 | 70 40 5 | 50 42 |
| JR-2-19 | 25 | 72 50 6 | 62 85 |
| MWS-14 | 42 | 0 5 NT | 40 35 |
| MWS-14-30 | 45 | 3 2 NT | 50 34 |
| MWS-14-34 | 46 | 3 1 NT | 45 31 |

* E/T cell ratio 10:1.
** Dilution of 1:100 of SPV-T3b, OKT4A, SPV-T8, and 1:400 of SPV-L1 and SPV-L5.
† Not tested.
autologous B lymphoma cells (MPL cells) is described. Panel studies with various cells from normal and malignant tissues, including many cells that are very susceptible for NK and AK cell killing, reveal that the CTL clone and its subclones preferentially lysed B lymphoma target cells. This autologous and allogeneic anti-lymphoma activity was not MHC-restricted and the cytotoxic reaction against the lymphoma cells was shown to be inhibited by a monoclonal antibody directed against LFA but not by antibodies against T3 and T4.

The CTL clone MWS-14 and its subclones were obtained by cocultivating the E+ fraction of the PBL from a B cell lymphoma patient with fresh autologous tumor cells, in primary and secondary MLTC followed by limiting dilution of the responder cells. Primary stimulation with the MPL cells did not result in the induction of cytotoxic activity (Table I). Restimulation, however, generated a cytotoxic reaction against the MPL cells, indicating that the PBL contained a small number of clones that could be enriched for cytotoxicity against the stimulator cell by repetitive stimulation. On the other hand, it cannot be excluded that suppressor cells present in tumor-bearing individuals (30, 31) were eliminated by these culture conditions.

We have shown previously that PBL of a melanoma patient that failed to kill
autologous short-term cultured melanoma cells, could be activated by cocultivation with the autologous melanoma cells, which resulted in the generation of cytotoxicity against autologous tumor cells (5). These CTL clones were shown to have a cytotoxic reactivity predominantly restricted to (short-term) cultured melanoma cells. Although it was demonstrated that the cytotoxic reactivity of the anti-melanoma CTL clones was not due to some form of AK activity, it could not be ruled out completely that culturing of the melanoma cells contributed in some way to the preferential cytotoxic reactivity against these cells.

However, similar CTL clones were obtained in the present study in which fresh noncultured lymphoma cells were used. These B lymphoma cells were shown to be insensitive for the activated T cell bulk cultures and a CTL clone with very high AK activity as measured against K562 cells. Moreover, MPL cells were only lysed by CTL clones, specific for those MHC determinants present on the lymphoma cell surface (HLA-A2, HLA-DRw6, and HLA-DC-1), whereas CTL clones directed at MHC determinants not present on the MPL cells did not lyse these cells. Given the fact that MPL cells appear to be sensitive for specific CTL, it seems fair to conclude that the cytotoxic activity of MWS-14 against autologous tumor cells is antigen specific. These results confirm and extend our previous findings obtained with CTL clones that preferentially lysed melanoma cells (5). The nature of the antigen(s) expressed on the tumor cells that are being recognized by MWS-14 remains to be determined. The panel studies show that MWS-14 and its subclones killed all B lymphoma target cells as far as tested. In contrast, only 2 or 3 of the 23 control cell lines were killed. Although a small panel of B lymphomas was tested, these data suggest that MWS-14 recognizes an antigen preferentially expressed on malignant B lymphoma cells. Normal autologous T blasts derived from the PBL of the B lymphoma patients were not killed, indicating that the target antigen is expressed only on the malignant cells. This notion is also supported by the finding that an EBV-transformed B cell line derived from the normal B cells of one donor (KOS) was not killed by MWS-14, whereas the B lymphoma cells of this donor were lysed efficiently. The determinant recognized by MWS-14 seems not to be restricted to B lymphoma cells, since 2, and 3, out of 23 nonrelated normal and malignant cell lines were killed by MWS-14-30 and MWS-14-34, respectively. The nature of this reactivity has to be investigated but it must be noted that the target cells killed by MWS-14 subclones (HPB-ALL, T24, and CEM) are occasionally also killed by CTL clones with well-defined allospecificities (reference 32; H. Spits, unpublished), especially when such allospecific CTL clones are in culture for a long period of time. Similar results have been reported by Brooks et al. (20), who showed that mouse allospecific CTL clones acquire natural killer cell–like activities after long periods of culture. Thus, the activity of MWS-14-30 against T24 and HPB-ALL and of MWS-14-34 against HPB-ALL, T24, and CEM may be due to acquired nonspecific killing activity.

In many immunological systems of both mouse and man, CTL activity against autologous target cells, in particular virus-infected autologous cells, has been demonstrated, and involves the recognition of the antigens in the context of self MHC determinants (33–36). HLA-restricted cytotoxicity can be blocked by monoclonal antibodies against MHC products (6, 7). The panel study that
demonstrated that MWS-14-killed B lymphoma cells, irrespective of the HLA phenotype of these cells, already indicated that the activity of MWS-14 is not restricted by polymorphic HLA determinants. This notion was supported by the finding that monoclonal antibodies, directed against monomorphic determinants present on class I and class II MHC antigens (which inhibited very efficiently the activity of allospecific CTL clones against MPL cells), failed to inhibit the activity of MWS-14 and MWS-14-34 against the MPL cells. In addition, the results of the blocking experiments made it unlikely that MWS-14 recognizes “alien” MHC antigens, which can be expressed on malignant cells (37-39). Our CTL clones differ from the lymphokine-activated killer (LAK) cells recently described by Grimm et al. (40, 41). Although these LAK also were capable of lysing NK-resistant fresh human tumor cells, they demonstrated a much broader specificity. In addition, these cells were all of the T8+ phenotype, whereas the anti-lymphoma CTL clones are T4+. However, the most important difference is that the anti-tumor cell reactivity of the LAK cells was established with bulk cultures, which allows no exact comparison with the CTL clones described here.

It has recently been shown that the T cell receptor for alloantigen is associated with T3 (28). Moreover a T3-positive NK clone has been shown to have a T cell receptor for non-MHC determinants present on K562 cells that is associated with T3 (42). However, the blocking studies using monoclonal antibodies against T3 (7, 26) and the modulation experiments with anti-T3 (Fig. 1) failed to demonstrate any involvement of T3 in the cytotoxic activity of MWS-14 against the MPL cells. These results are similar to those obtained in the melanoma system, in which it was demonstrated that T3 was not involved in the preferential cytotoxic activity of CTL clones against autologous melanoma cells (5). The data presented here and in the previous paper (5) indicate that a novel type of cytotoxic activity, which is antigen specific, but not associated with T3, can be generated in vitro.

Monoclonal antibodies to the T4 glycoprotein have been shown to be able to block the cytotoxic activity of T4+ CTL (26, 27). In spite of the expression of T4 on CTL clone MWS-14, blocking studies with anti-T4 reagents indicated that T4 is not involved in the cytotoxic activity of this clone against MPL. This is not surprising because T4 seems to play a role in class II MHC-restricted cytotoxic reactions only, and the involvement of class II MHC antigens in the activity of MWS-14 against MPL could not be demonstrated. It has been hypothesized that the T4 structure might serve as a stabilizer of the adhesion of the CTL to the target cell in the case of T4+ CTL with a low affinity for their target antigen (26, 43). Therefore, the failure of OKT-4A to block the cytotoxic reaction of MWS-14 against MPL might also be explained by assuming that this clone has a high affinity for the autologous B lymphoma cells.

LFA-1 is thought to represent an adhesion structure that is important for cell-cell contact (16). Antibodies against LFA-1 block antigen-specific (2, 17, 29), NK (12, 29), AK (12), K cell activity (F. Miedema, submitted for publication), and lectin-dependent cytotoxicity (12). By preventing conjugate formation between the effector and target cells (H. Spits and R. Roozendaal, unpublished observations). It is shown (Table VI) that LFA-1, as defined by the monoclonal antibodies SPV-L1 and SPV-L5, also plays a role in the auto-tumor CTL activity of MWS-
14, indicating that cell-cell contact is indeed required for the cytotoxicity me-
diated by clone MWS-14 to occur.

Experiments to investigate the nature of the antigen on the B lymphoma cells
recognized by the CTL clones are currently in progress.

Summary
A human cytotoxic T cell clone (MWS-14) with auto-tumor reactivity was
established in serum-free medium in a mixed tumor cell culture by repetitive
stimulation with fresh autologous lymphoma cells. This clone and its subclones
are of the T3+ T4+ T8- phenotype. They were strongly cytotoxic for the
autologous lymphoma cells, whereas autologous PHA blasts were not killed.
Analysis of the specificity of MWS-14, MWS-14-30, and MWS-14-34 indicated
that these CTL clones were cytotoxic for 7/7 allogeneic lymphoma cells, whereas
only 3/23 of normal and non-lymphoma cells were lysed. Blocking studies with
monoclonal antibodies directed at MHC class I and class II antigens showed that
this preferential, anti-lymphoma reactivity was not directed at HLA determinants.
The anti-lymphoma activity is not due to an aspecific susceptibility of the
lymphoma cells to lysis. In contrast to CTL clones specific for HLA antigens
present on the lymphoma cells, T3 and T4 were not involved in the cytotoxic
reaction of MWS-14 against the autologous lymphoma cells. The reactivity of
this clone could be blocked by a monoclonal antibody directed at leukocyte
function-associated antigen.

It can be concluded from these results that these T4+ CTL clones recognize
a determinant, which is preferentially expressed on autologous and allogeneic
lymphoma cells.

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