Lack of T-Cell-mediated Recognition of the Fusion Region of the pml/RAR-α Hybrid Protein by Lymphocytes of Acute Promyelocytic Leukemia Patients

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ABSTRACT

In previous studies, it was shown that the fusion region of the pml/RAR-α protein, expressed by acute promyelocytic leukemia (APL) cells, can be specifically recognized in vitro by donor (D. E.) CD4 T cells in a HLA class II DR1-restricted fashion. We present here the results on the recognition of several pml/RAR-α peptides by APL patients expressing HLA DR1. The in vitro immunization of peripheral blood lymphocytes from four patients in remission (S. R., F. R., M. M., P. G.) with BCR1/25, a 25-mer pml/RAR-α, did not elicit either a polyclonal or a clonal immune response specific to the peptide. We then generated new donor anti-pml/RAR-α CD4+ T-cell clones. These clones were tested for their recognition of BCR1/25. One clone (C3/5, CD3+, CD4+, CD8−) was selected for further analysis. Clone C3/5 showed specific proliferation, cytotoxicity, and cytokine (tumor necrosis factor α, granulocyte-macrophage colony-stimulating factor) production when challenged with autologous lymphoblastic cell lines pulsed with peptide BCR1/25. C3/5 cells developed specific proliferation and cytotoxicity when challenged with peptide-pulsed lymphoblastic cell lines and peripheral blood lymphocytes from the four DR1+ APL patients. APL blasts, available only from patients F. R. and P. G., were not lysed by C3/5 and were unable to present peptide BCR1/25. Incubation of APL cells with IFN-γ failed to induce HLA class II molecules and recognition by the C3/5 clone. Since APL cells do not express HLA class II molecules, we tested in two donors (D. E. and C. H. R.) and in patients S. R. and P. G. whether the use of 9-mer peptides (BCR1/9) would generate a CD8+HLA class I-restricted response. No peptide-specific T-cell line or clone could be generated from both donors and patients. These findings are discussed in relation to possible therapeutic approaches to the immunotherapy of APL.

INTRODUCTION

The pml/RAR-α fusion protein, specifically expressed in APL (1-4) cells, creates a new tumor-specific amino acid sequence. It has been shown that a 25-mer peptide (BCR1/25), encompassing the fusion region, contains an antigenic site (absent in the normal parent molecules) recognizable by CD4+ T-cell clones of a healthy donor (D. E.) in an HLA DR1-restricted fashion on presentation by autologous APCs (5, 6). Antipeptide CD4 clones also recognize pml/RAR-α-transfected LCLs (6).

The possible recognition of the pml/RAR-α protein by T lymphocytes of APL patients would open the possibility of directing the patient’s immune system against the leukemic cells through the recognition of a tumor-specific/transformation-related molecule (5). Although binding motifs for most DR molecules were not known at the time of the study, our previous data (6) indicated that DR1 functions as a restriction element in this case. For this reason, we focused on the analysis of DR1+ of DR11 APL patients.

In the present work, we studied the in vitro immune response of peripheral blood lymphocytes from four HLA DR1+ APL patients to several pml/RAR-α peptides.

PATIENTS AND METHODS

Patients

Four APL patients were studied after informed consent was obtained. Three patients (F. R., S. R., and P. G.) were in first remission, and one patient (M. M.) was in second remission. Two patients (S. R. and M. M.) underwent autologous bone marrow transplantation: the conditioning regimen included high-dose chemotherapy but not total-body irradiation. At the time of the study, patients had not received treatment for at least 6 months, and their peripheral counts were within normal ranges.

1 The abbreviations used are: APL, acute promyelocytic leukemia; LCL, lymphoblastoid cell line; APC, antigen-presenting cell; PBMC, peripheral blood mononuclear cell; LAK, lymphokine-activated killer; IL, interleukin; HPLC, high-performance liquid chromatography; HS, human serum; dThd, thymidine; PBL, peripheral blood lymphocyte; TNF-α, tumor necrosis factor α; GM-CSF, granulocyte-macrophage colony-stimulating factor; MAb, monoclonal antibody; PHA, phytohemagglutinin; TT, tetanus toxoid; SI, stimulation index; TCR, T-cell receptor.
**PBMCs**

Peripheral blood was obtained from two healthy donors (D. E. and C. H. R.) and four APL patients in remission (S. R., F. R., M. M., and P. G.) after informed consent. PBMCs were isolated by centrifugation on Ficoll gradients. The HLA typing was as follows: DE (A2,23, B49,35, DR11,13), CHR (A2,11, B35,60, DR1,4), SR (A1,24, B51, DR8,11), FR (A23, B49,51, DR4,11), MM (A23,10, B27,49, DR10,11), and PG (A1,2, B15, DR1,11).

**Cell Lines**

Lymphoblastoid cell lines (DE.LCL, SR.LCL, FR.LCL, MM.LCL, PG.LCL, and CHR.LCL) were prepared from PBMCs as previously described (6). LAK cell lines were prepared by culturing PBMCs with recombinant IL-2 (10³ units/ml; Euro-Cetus BV, Amsterdam, the Netherlands). The Daudi cell line was cultured in RPMI 1640 (Biowhittaker, Walkersville, MD) plus 10% FCS (Biological Industries, Kibbutz Beth Haemek, Israel). Fr.APL cells were obtained from bone marrow or peripheral blood of an APL patient (F. R.) and frozen in liquid nitrogen. They were thawed and cultured for 18 to 24 h before use in proliferation or cytotoxicity tests.

**Peptides**

The following peptides were used: BCRI/25, a 25-mer (NSNVASGAGEAAIETQSSSSSEEIV) peptide, and a pool of nine different 9-mer peptides (VAGSAGEAA, ASGAGEAAL, SGAGEAAIE, GAGEAIEIT, AGEAIAETQ, GEAIAETQS, EAAIETQSS, AAATGQQSS, and AATETQSSS) collectively indicated as BCRI/9. They encompass the BCRI-type fusion region of the pml/RAR-α protein (4). The peptides were synthesized by the University of Wisconsin Biotechnology Center (Madison, WI) or American Peptide Company (Sunnyvale, CA) and purified using high-performance liquid chromatography to a minimum purity of 95%.

**Activation of Lymphocytes**

The protocol described by Chen et al. (7) was followed. In brief, 50 × 10⁶ fresh PBMCs (10⁸ for patient P. G. and donor C. H. R.) were incubated in a humidified atmosphere at 37°C in 5% CO₂ with 20 μM corresponding peptides at 5 × 10⁶ cells/ml serum-free RPMI 1640 medium for 1 h. Fresh autologous PBMCs (50 × 10⁶ cells in 10 ml RPMI 1640 medium + 10% HS) were added to each APC culture in culture flasks (75 cm²). The flasks were incubated upright for 7 days. Primed lymphocytes were then harvested and restimulated with irradiated (3000 rad) autologous PBMC-pulsed peptides (20 μM) at a lymphocyte: APC ratio of 1:3. The next day, IL-2 (10 units/ml) was added, and the percentage of HS was increased to 10%. Five days later, one half of the medium from each culture was replaced with RPMI 1640 + 10% HS + 10 units/ml IL-2. The derived T-cell lines were left in culture for 7 days before cloning on day 21 poststimulation. They were maintained in culture by periodic stimulation (every 7–10 days) using irradiated (8000 rad) autologous LCL-pulsed peptide (20 μM) and IL-2 (10 units/ml). To test for proliferative activity of the derived T-cell lines, cells were washed and placed in U-bottomed 96-well plates (5 × 10⁵ cells/well). They were stimulated with irradiated (8000 rad) autologous LCLs (10⁵ cells/well) in the presence or absence of the appropriate peptides. LCL cells were washed three times in serum-free RPMI 1640 medium, incubated with 20 μM peptides as indicated above, and then irradiated. After 48 h of culture, the plates were labeled with [³H]dThd, further cultured for 18 h, harvested, and counted in a beta counter.

**Generation of Lymphocyte Clones**

Primed T-cell lines were cloned on day 21 by limiting dilution (20, 10, 5, 2, and 1 cell/well/200 μl RPMI 1640 + 10% HS) in 96-well U-bottomed plates. Autologous LCLs, PBLs, and allogeneic PBLs (25 × 10³ cells/well) were used as a feeder layer. Autologous PBLs and LCLs were incubated separately in serum-free RPMI 1640 medium (5 × 10⁶ cells/ml) with 20 μM of the same mixture of peptides, used to prime lymphocytes (see above), for 30 min at 37°C before irradiation (3000 rad for PBLs and 8000 rad for LCL cells). X-irradiated (3000 rad) allogeneic PBLs and IL-2 (50 units/ml) were also added to the wells. The plates were incubated for 14 days; 100 μl fresh medium and 50 units/ml IL-2 were replaced in each well every 3 days. Wells with positive signs of growth were selected for expansion (see “Results”). The probability of clonality for each well containing growth was calculated as described previously (6). Growing clones were transferred to 96-well flat-bottomed plates, and each clone was stimulated with 25 × 10⁶ irradiated (8000 rad) autologous LCLs pulsed with the appropriate peptides (20 μM) and 25 × 10⁵ (3000 rad) irradiated allogeneic PBLs. IL-2 (50 units/ml) was added to the wells after 1 day. This activation cycle was repeated after 7–10 days of culture at lymphocyte: autologous LCL:allogeneic PBL ratios of 1:1:1, unless otherwise indicated.

**Screening of Clones**

The following assays were carried out.

**Proliferation Assay.** T-cell clones were first screened for proliferative activity 28 days after cloning. Each well was washed off to remove IL-2 and split into three. The first one was expanded for further culture. The second well was stimulated with irradiated (8000 rad) autologous LCLs (10⁵ cells/well) pulsed with the appropriate peptides (20 μM). The third well was incubated with irradiated LCLs without peptides. Cells were cultured for 48 h, incubated for an additional 18 h with [³H]dThd (1 μCi/well), and harvested. In subsequent experiments, the incubation time was reduced to 24 h (see “Results”).

**Cytotoxic Assay.** T-cell clones were first screened for cytotoxic activity 6 weeks after cloning; one third of the cell suspension from each clone was admixed with ⁵¹Cr-labeled autologous LCLs (10⁶ cell/well) as a control, and the other third was incubated with LCLs pulsed with peptide (20 μM). The assay was performed as previously described (6). The incubation time was 4–6 h. Spontaneous release never exceeded 15%. Anti-TNF-α monoclonal antibody (Farmitalia, Milan, Italy) and TNF-α (EuroCetus BV) were used in some assays (see “Results”).

**Cytokine Production and Detection Assays**

Cells from clone C3/5 (3 × 10⁶ cells) were cultured in 48-well plates (1.5 × 10⁶ cells/ml) with irradiated (8000 rad) autologous DE.LCL (3 × 10⁶ cells) in the presence or absence
of 20 μM peptide (25-mer). C3/5 cells (3 × 10⁶) were also cultured in 2 ml RPMI 1640 + 10% HS in the absence of LCLs. The supernatant from each culture (2 ml) was collected by centrifugation after 1 and 2 days, immediately frozen in liquid nitrogen, and stored at −80°C until used. The cytokines assayed in the present study were: IL-2, IL-3, IL-4, IL-6, IFN-γ, TNF-α, and GM-CSF. IL-2 activity was measured by [3H]dThd uptake of IL-2-dependent cytotoxic T lymphocyte line cells as previously described (Ref. 8, 1 unit biological activity was referred as half-maximal proliferation of cytotoxic T lymphocyte line cells). In brief, serial dilutions of IL-2 or supernatant from each culture were incubated in 200 μl for 24 h, followed by an overnight pulse of 1 μCi/well of [3H]dThd. IL-3 was assayed by using a Quantikine Human IL-3 Immunoassay (Research and Diagnostic Systems, Minneapolis, MN) with a detection limit of 30 pg/ml. IL-4 and IFN-γ were measured using the sandwich ELISA as described in detail elsewhere (9). The mouse MAb 8F12 (10) and biotinylated 3H4, generously provided by C. H. Heusser (Ciba-Geigy Ltd., Basel, Switzerland), were used for IL-4, and MAbS 43-11 and biotinylated 45-15, kindly supplied by S. Alkan (Ciba-Geigy Ltd.), were used for IFN-γ. The sensitivities of the IL-4 and IFN-γ ELISA were 30 and 40 pg/ml, respectively. IL-6 and TNF-α were analyzed using kits from Chromogenix AB (Molndal, Sweden), with a detection limit of 4 pg/ml as given by the manufacturer. The human GM-CSF was assayed using Immotest human GM-CSF from Innogenetics NV (Antwerp, Belgium) with a detection limit of 8 pg/ml.

**PHA and TT Assays**

PBLs were simultaneously thawed, washed, and seeded at 10⁵ cells/well. PHA (Murex, Temple Hile, England) was added at 1 μg/ml, and proliferation was assessed as [3H]dThd uptake (6-h incubation) after 72 h. For proliferation to TT (10 μg/ml; Connaugh Lab), 2 × 10⁵ PBLs/well were cultured for 6 days and labeled with [3H]dThd for the last 18 h of culture.

**Immunofluorescence Assay**

This assay was performed as previously described (11). The MAbs used were in the form of diluted ascites: anti-CD3, anti-CD4, anti-CD8, anti-TCR-β, anti-TCR-γδ, anti-HLA class I (W6/32), and anti-HLA class II (D1-12). Samples were analyzed using flow cytometry (FACs IV; Becton Dickinson, Sunnyvale, CA) with a logarithmic signal amplification.

**Screening for HLA-binding Motifs**

A list including the binding motifs for 35 HLA class I specificities was kindly supplied by Dr. H. G. Rammensee (Heidelberg, Germany) and includes the following specificities: A1, 201/5/3, 3, 11, 24, 31, 33, 68; B7, 8, 2702/5, 3501/3, 3701, 3901/2, 40, 4402/3, 5102/3, 5201, 53, 58, 60, 61, 62, 78; Cw301, 0401, 0602, 0702.

**HLA Stabilization Assay**

The ability of peptides to bind HLA molecules was assessed by the peptide-mediated increase in the amount of HLA expression on the membrane of the T2 (12), L721.221(13), and ST-EMO (14) cell lines. Cw6 and Cw0702 transfectants of T2 and L721.221 were also used (kindly supplied by Dr. D. Schendel, Munich Germany). Cells were washed in serum-free RPMI 1640 and incubated with 20–100 μg/ml peptide for 18 h at 37°C. Expression of HLA class I molecules was evaluated by indirect immunofluorescence with W6/32 MAb or with monospecific MAbs GAP-A3, 4E (anti-B/C), or BB7.2 (anti-A2). Positivity was defined as an increase by at least 50% in the median channel of fluorescence.

**RESULTS**

**Polyclonal T-Cell Lines.** The results for the proliferative activity of T-cell lines, obtained from an APL patient in remission (S. R.) and a healthy donor (D. E.), are presented in Fig. 1. SR/9 and SR/25 were obtained by culture in the presence of BCR1/9 or BCR1/25; DE/9/25 was derived from cultures in the presence of both BCR1/9 and BCR1/25 peptides. SR T-cell lines did not show a specific proliferative activity against autologous peptide-pulsed LCLs. A low but reproducible SI (SI = cpm test well/cpm control well) of 2.2 was obtained for the DE/9/25 T-cell line. These lines consisted of a mixture of CD4 and CD8-α/β lymphocytes. No specific cytotoxic activity against peptide-pulsed LCLs was obtained in these cell lines (data not shown).

**T-Cell Clones.** The T-cell lines SR/9, SR/25, and DE/9/25 were cloned by limiting dilution. Two hundred fifteen clones (with a clonal probability of >90%) were obtained; 52, 41, and 122 clones were generated from SR/9, SR/25, and
Immune Recognition of the pml/RAR-α Protein

DE/9/25, respectively. Among the 52 SR/9 and the 41 SR/25 clones tested, no specific cytotoxic or proliferative activity was obtained. Proliferation against LCLs pulsed with control peptide or with PBS was observed in several clones, suggesting autoreactivity or anti-EBV reactivity (data not shown). Among the 122 clones obtained from the DE/9/25 T-cell line, 59 were autoreactivity or anti-EBV reactivity (data not shown). Among the 52 SR/9 and the 41 SR/25 clones tested, no specific cytotoxic or proliferative activity was observed, while a specific response was obtained from donors' PBLs (all being HLA-A2) in response to the influenza peptide GILGFVFTL (data not shown).

Specific Cytokine Production by the C3/5 Clone in Response to Autologous Peptide-pulsed LCLs. To assess cytokine production, cells from the C3/5 clone were stimulated with autologous DE.LCL in the presence or absence of the BCR1/25 peptide (20 μM), and cytokine production was measured after 1 and 2 days of culture. IL-2, IL-3, IL-4, IL-6, IFN-γ, TNF-α, and GM-CSF were assayed. IL-2, IL-3, IL-4, IL-6, and IFN-γ levels were undetectable or below the detection limit of the assay used (data not shown). C3/5 cells cultured with autologous DE.LCL-pulsed peptide for 1 day secreted significant amounts of TNF-α (933, 1189 pg/ml) and GM-CSF (3395, 3301 pg/ml) in two separate experiments compared with those stimulated with DE.LCL in the absence of peptide (26 pg/ml or below the detection limit for TNF-α and 184, 126 pg/ml for GM-CSF). TNF-α and GM-CSF production peaked at day 1 and remained stable at day 2 (Fig. 3). To test whether TNF-α produced by the C3/5 clone could be involved in its cytolytic activity, TNF-α and anti-TNF-α MAb were used. The addition of anti-TNF-α at different concentrations 0.1, 1, and 10 μg/ml to the target cells DE.LCL in the presence or absence of the peptide, did not inhibit the cytolytic activity exerted by the C3/5 clone (Fig. 4). The antibody was instead active in inhibiting the cytotoxic activity of TNF-α against the TNF-α-sensitive WEHI 164 cell line (data not shown). TNF-α, at 1–1000 units/ml, had no cytolytic activity on DE.LCL cells when added for the same

Fig. 2 Cytotoxic activity of clone C3/5 (E. effector cells) against autologous DE.LCL (T, target cells) in the presence of BCR1/9 (B) and BCR1/25 (C) or absence (A) of peptide. LCLs at 5 × 10⁶ cells/ml were incubated with ⁵¹Cr for 90 min with or without peptides (20 μM). After washing, the peptides were readded to the target (10⁴ cells/well) at the same concentration.

Fig. 3 Specific cytokine production by the C3/5 clone. Cells (1.5 × 10⁶/ml) were cultured with irradiated (8000 rad) autologous DE.LCL (1.5 × 10⁶ cells/ml) in the presence (■ and ▼) or absence (○ and ▲) of the BCR1/25 peptide (20 μM). Supernatants were harvested on days 1 and 2 and assayed for the presence of TNF-α (■ and ●) and GM-CSF (▼ and ▼).

Cytotoxic activity of clone C3/5 (E. effector cells) against autologous DE.LCL (T, target cells) in the presence of BCR1/9 (B) and BCR1/25 (C) or absence (A) of peptide. LCLs at 5 × 10⁶ cells/ml were incubated with ⁵¹Cr for 90 min with or without peptides (20 μM). After washing, the peptides were readded to the target (10⁴ cells/well) at the same concentration.
Fig. 4  Effect of MAb directed against TNF-α produced by the C3/5 clone on the cytotoxic activity. Cells from the C3/5 clone were challenged with autologous DE.LCL in the absence of peptide (0) and in the presence of 20 μM BCR1/25 peptide (●). Anti-TNF-α at 0.1 μg/ml (▽), 1 μg/ml (▼), and 10 μg/ml (▲) was mixed with LCL-pulsed peptide (BCR1/25) before the effector cells were added.

duration of the cytotoxic assay (data not shown). The results indicate that cytotoxic activity and TNF-α release probably represent two separate functions in clone C3/5, although both are specifically induced by BCR1/25.

Reactivity of C3/5 toward HLA-DR1 Allogeneic APLs, LCLs, and PBLs. Five allogeneic LCLs [SR, FR, MM, PG (sharing DR11 with DE and obtained from APL patients) and CHR (DR11⁺)] were subjected to a cytotoxic test in the presence or absence of BCR1/25 (20 μM) at different E:T ratios. The results are shown in Fig. 5. The C3/5 clone specifically lysed DR11⁺matched SR, FR, and MM [and PG (data not shown)] LCLs pulsed with BCR1/25, as well as the autologous DE.LCL. CHR cells failed to present the peptide to the C3/5 clone. The ability of the C3/5 clone to proliferate to the autologous DE.LCL and HLA-DR11-matched SR, FR, MM, and PG.LCL pulsed with different peptide concentrations was also investigated. A preliminary experiment was carried out to determine the optimal stimulation period giving the highest proliferative activity of clone C3/5 at this stage of culture. Cells were cultured for 24, 48, and 72 h with autologous irradiated (8000 rad) DE.LCL in the presence (2 and 20 μM) or absence of the BCR1/25 peptide, incubated for an additional 18 h with [3H]dTdt, and harvested. As shown in Fig. 6A, the 24 h time point gave the highest cpm count (P < 0.05 using the t test analysis). The proliferative activity of the C3/5 clone against autologous and allogeneic LCL-pulsed peptide (0, 5, 20, and 50 μM) was subsequently studied at the 24-h time point (Fig. 6B). All DR11⁺ peptide-pulsed LCLs stimulated the C3/5 clone in a dose-dependent fashion (PG.PBL induced an uptake of 38,500 ± 2,300 cpm). Although some discrepancies between proliferation and cytotoxicity were observed (SR and MM.LCL induced a good proliferative response but produced low levels of cytotoxicity, while FR.LCL induced little proliferation but was very efficiently lysed by C3/5), all four APL patients’ LCLs were able to present peptide BCR1/25 to C3/5. The ability of LCLs derived from APL patients to present peptide BCR1/25 to C3/5 is suggested that the failure in raising anti-pm1IRAR-ct clones from the four APL patients was probably due to a defect in antigen presentation capability. To further investigate this point, this experiment was replicated using patients’ PBLs (instead of LCLs) as APCs. The results are presented in Table 1, and confirm that the antigen presentation capability in the APL patients studied was grossly intact.

Since the C3/5 clone was specifically able to lyse FR.LCL cells pulsed with the BCR1/25 peptide in a HLA class II (DR11) restriction fashion, we further tested whether this clone could exert a cytotoxic activity against FR.APL cells. Because APL cells are usually DR₁⁺, FR blasts (≥85% leukemic cells) were also cultured with IFN-γ (a cytokine known to induce or enhance the expression of HLA class II molecules) before being used. The results are present in Fig. 7. C3/5 cells did not lyse FR.APL blasts and the culture of FR.APL cells with IFN-γ (10³ units/ml) for 48 h before the experiment did not enhance the cytolytic activity. This was possibly due to the failure of IFN-γ to induce HLA class II molecules on FR.APL blasts as assayed by immunofluorescence (data not shown). FR.APL cells were also unable to present peptide BCR1/25 even after IFN-γ pre-incubation. To test whether the interaction of C3/5 cells with peptide-pulsed FR.LCL could kill the APL blasts by a bystander
Table 1  Antigen presentation capability of APL patients’ PBL

<table>
<thead>
<tr>
<th>Type of APCs</th>
<th>+ BCR 1/25</th>
<th>− BCR 1/25</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE.LCL</td>
<td>2.5 ± 0.5</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>DE.PBL</td>
<td>18.0 ± 2.6</td>
<td>6.0 ± 0.6</td>
</tr>
<tr>
<td>SR.PBL</td>
<td>16.5 ± 3.9</td>
<td>5.6 ± 0.9</td>
</tr>
<tr>
<td>FR.PBL</td>
<td>18.6 ± 1.1</td>
<td>4.6 ± 0.8</td>
</tr>
<tr>
<td>MM.PBL</td>
<td>15.2 ± 3.7</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>PG.PBL</td>
<td>18.0 ± 1.8</td>
<td>3.5 ± 0.2</td>
</tr>
</tbody>
</table>

*C3/5 cells (50000/well) were cocultured with 10^5 irradiated APCs incubated with BCR 1/25 (25 μM) or PBS. Plates were cultured for 48 h, labeled with [3H]dThd for additional 18 h, and harvested. Values represent the mean × SD cpm obtained in three replicates. C/S cells incubated in medium alone incorporated 195 ± 70 cpm; irradiated APCs incorporated 419-841 cpm.

**Fig. 6** Proliferative response of the C3/5 clone against the BCR1/25 peptide. In A, the C3/5 clone (5 × 10^4 cells/well) was cultured for 24, 48, and 72 h with irradiated (8000 rad) autologous DE.LCL (10^5 cells/well) in the absence (□) or presence of 2 μM (■) and 20 μM (□) of peptide BCR1/25 (D), lymphocytes cultured in medium alone. In B, the C3/5 clone (5 × 10^4 cells/well) was cultured for 24 h with irradiated (8000 rad) autologous DE.LCL or allogeneic SR.LCL, FR.LCL, and MM.LCL (10^5 cells/well) in the presence of 5 μM (□), 20 μM (□), and 50 μM (□) or in the absence (□) of BCR1/25 peptide. LCLs at 5 × 10^6 cells/ml were incubated with the peptide for 30 min before irradiation, cultured for an additional 18 h with [3H]dThd, and harvested. cpm values represent the means of three replicates. Bars, SD.

**Fig. 7** Cytotoxic activity of clone C3/5 against allogeneic FR.APL blasts before and after incubation with IFN-γ (10^4 units/ml, 48 h) in the presence (○, △, ▽, and △) or absence (●, □, ▽, and ▽) of the BCR1/25 peptide. The target cells used were: FR.LCL (○ and □), FR.APL (○ and ▽), FR.APL + FR.LCL (△ and △), FR.APL + IFN-γ (△ and △), and LAK + FR.APL (○).

**PHA and TT Responses of Patients’ PBLs.** Because of the negative results obtained with lymphocytes from APL patients, a PHA response assay was performed to test the T-cell response in these patients. Results are presented in Table 2 and show that, compared to PBLs from two normal donors (D. E. and C. H. R.), patients’ PBLs showed a dramatically reduced response. These results were surprising to us, since all patients were studied while in remission and off treatment for >6 months (F. R. was in complete remission and off therapy for >2 years). Comparable results were obtained when response to a specific antigen (TT) was evaluated. Six-day proliferative responses to TT averaged 293,959 ± 42,793 (SD) cpm in three donors (including D. E. and C. H. R.) and 49,602 ± 15,047 cpm in the patient group (P = 0.002).
Finally, LCLs and PBLs derived from APL patients expressing the relevant restriction element (HLA-DR11) are able to present BCR1/25 similar to autologous LCLs.

On the other side, two different sets of data have to be considered. First, repeated attempts at generating anti-BCR1/25 T-cell clones in four DR1+ APL patients were not successful. These results cannot be attributed to an insufficient antigen-presenting capability in these patients, since their LCLs and PBLs induced proliferation and cytotoxicity in the C35 clone in the presence of BCR1/25 similar to autologous LCLs. However, it is possible that the frequency and/or the responsiveness of anti-BCR1/25 lymphocytes was particularly low in these patients, even if PBLs were recovered when the patients were in remission with normal peripheral blood counts and T-cell subsets values. The results of the PHA and TT response assays strongly support this conclusion and indicate that a generalized impairment of the cellular immune system, already reported in cancer patients (15), can be present even during long-term remissions. The molecular analysis of the observed hyporesponsiveness is presently under investigation in our laboratory.

The possible deletion or suppression of anti-pml/RAR-α-specific T-cell clones by APL cells is another possibility that deserves further investigation as well as ways to increase the in vivo frequency of anti-pml/RAR-α precursors in APL patients. The failure to generate anti-BCR1/25-specific clones in APL patients represents the most relevant problem to be solved before therapeutic approaches can be considered.

Second, in both APL patients and healthy donors we were unable to raise CD8, HLA class-I restricted T-cell clones. This is of particular importance given the usual and strong suppression of the DR gene expression in APL cells. Although our strategy of pulsing APCs with short peptides could not have been successful for technical reasons, it produced positive results in other systems (16) and against known immunogenic peptides. It has been demonstrated that inhibitory peptides competitively block the binding to HLA molecules of stimulatory peptides (17). Since a mixture of 9-mer peptides was used in our experiments, peptide cross-inhibition cannot be excluded. The use of different stimulation protocols (e.g., using pml/RAR-α-transfected cells) or APCs (like dendritic cells) could produce positive results. These results can also be explained by the observed lack of effective binding motifs for HLA class I molecules in the pml/RAR-α junction. A recent article by Bocchia et al. (18), analyzing HLA binding to two pml/RAR-α peptides, is consistent with such an hypothesis.

Thus, it is possible that in APL, the pml/RAR-α protein is recognized by T lymphocytes, but only when presented by a restriction element (HLA-DR11) that is not present on the APL cells themselves. The recognition of putative DR* APL stem cells in the form of colony-forming units could still be possible. However, the existence of these cells has yet to be demonstrated (19), and true APL colonies are difficult to obtain (20). Alternatively, the induction of DR molecules on APL cells could permit an efficient immune recognition (20).

The lack of expression of DR molecules by APL cells and the resulting inability of DR11* APL cells to present pml/RAR-α peptides could also be reflected by the lack of clinical significance of DR11 expression in APL patients undergoing bone marrow transplantation. In collaboration with the Interna-
nitional Bone Marrow Transplant Registry, an analysis was conducted in 189 APL patients in first remission who underwent an allelogeneic related bone marrow transplant. The patients were grouped into HLA-DR11* (n = 44) and HLA-DR11+ (n = 145). No significant difference in survival or disease-free survival was evident between the two groups. Also, the proportion of DR11 patients (23%) does not differ significantly from that seen in the general population. In case the pml/RAR-α junction could not be presented by the HLA class I molecule, then the only chance that this fusion protein can be recognized in APL cells by T lymphocytes resides in the restoration of DR expression through pharmacological (21) or genetic manipulation, or in the targeting of the still hypothetical DR- APL stem cells.

Our studies demonstrate that the BCR1/25 (peptide corresponding to the fusion region of the pml/RAR-α protein present in APL cells can be specifically recognized by donor (but not by patient) CD4 T cells in a HLA class II (DR11)-restricted fashion; CD8/HLA class I restricted T cells were not generated, possibly due to the lack of HLA-binding motifs in the pml/RAR-α fusion region. Finally, APL blasts were not lysed by anti-pml/RAR-α cytotoxic T cell clones due to the lack of expression of the DR11 molecule by the APL blasts studied. Further immunotherapeutic approaches to APL will need to overcome the two main problems encountered in this study: the lack of DR expression in APL cells (20) and the poor immune status of APL patients.

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