Differential Recognition of a BCR/ABL Peptide by Lymphocytes from Normal Donors and Chronic Myeloid Leukemia Patients

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ABSTRACT

The BCR/ABL oncogenic fusion protein transforms normal bone marrow stem cells into neoplastic cells. It has been shown that peptides derived from the junctional region of this oncogenic fusion protein can be recognized by human T-lymphocytes obtained from normal donors. In this study, we investigated the immunogenicity in patients with chronic myeloid leukemia (CML) of a 17 mer b3/a2 Bcr/abl peptide (B/A1), which was shown to induce proliferative responses in lymphocytes from normal donors.

A total of 56 CML patients in chronic phase were studied. Twenty-two patients were studied at diagnosis without any treatment (group I). Fourteen patients were receiving IFN (group II), 14 patients were being treated with hydroxyurea (group III), and 6 patients were on different regimens (group IV). Patients were initially assessed for general immunological competence using both in vivo and in vitro assays. Patients were also selected for the expression of HLA-DR0401, the HLA specificity known to present peptide B/A1 to CD4 lymphocytes.

With the exception of the six patients in group IV, the results of all these assays (in vitro phytohemagglutinin/tetanus toxoid responses, in vivo skin reaction to ubiquitous antigens) in CML patients did not significantly differ from those obtained in normal donors, thus excluding the presence of generalized immunosuppression. Eight patients with HLA-DR0401 and a b3/a2 type of fusion were identified and further studied. In these eight patients dendritic cells were obtained from adherent peripheral blood mononuclear cells and used to stimulate CD4 lymphocytes. No patient developed a specific response to the bcr/abl peptide, although patients’ lymphocytes proliferated in response to a promiscuous tetanus toxoid peptide in all but one case. In contrast, response to the bcr/abl peptide was observed in seven of eight HLA-DR0401 healthy donors tested. These data suggest that immunocompetent, HLA-DR0401+ CML patients are unable to respond to peptide B/A1, at difference from healthy donors. The implication of these results for the immunotherapy of CML is discussed.

INTRODUCTION

CML represents one of the earliest examples of a neoplastic disease in which the molecular events leading to transformation have been characterized. The 9:22 chromosomal translocation generates hybrid genes named BCR/ABL, which code for an OFP with tyrosine kinase activity. Although additional genetic lesions are needed for CML to progress to blast crisis and to convert to an acute leukemia, the 9:22 translocation is considered to be both necessary and sufficient to cause the chronic phase of the disease. This knowledge has increased our ability to diagnose CML and to monitor treated patients in remission, but has not yet been translated into better treatment modalities. The only curative treatment for CML remains allogeneic bone marrow transplantation, a costly and toxic procedure, which, for various reasons, can be performed in less than 15% of patients. As in every OFP, the fusion region of the protein codes for a unique structure (1, 2). Therefore, peptides encompassing this area could be recognized by T-lymphocytes, if they are able to bind and be presented by HLA molecules. Like many oncogenic proteins, bcr/abl is not a membrane-bound protein; this fact could hinder this potentially immunogenic protein from being recognized by the immune system. However, the intracellular location of the protein is not detrimental for its recognition; it is known that intracellular proteins can undergo partial digestion within the cell, with the resulting peptides being presented in association with HLA molecules on the cell membrane. Both cytoplasmic, mitochondrial, and nuclear proteins can be recognized by the immune system through this mechanism (3–5). In fact, the antigens cloned from the tum− variants of the P815 mouse mastocytoma have been shown to be intracellular proteins (3), as most human melanoma antigens identified thus far are.
An important preliminary information is represented by the knowledge of what HLA-binding motifs are present inside the BCR/ABL fusions. Our group mapped the class I HLA-binding motifs for 44 OFFs (6). Common motifs are present in all CML subtypes, with p190 and the b3/a2 form of p210 being the two types with the most frequent binding motifs. In addition, several groups reported that peptides encompassing the bcrabl fusion region and presenting HLA-binding motifs can, indeed, bind HLA molecules and can be recognized by both CD8 and CD4 lymphocytes (7–11). These peptide-specific lymphocytes have been shown in some, but not all, instances to cross-react with CML cells expressing the relevant HLA specificity and the appropriate bcr/abl fusion type.

With a single exception (10), all these data have been obtained using healthy donor lymphocytes, and almost no information is available on the ability of effectors obtained from CML patients to recognize bcr/abl-derived peptides. The difficulty in obtaining patient data in this particular disease stems, in part, from the usually low percentage of lymphocytes in the mononuclear fractions isolated from the peripheral blood, due to the massive presence of leukemic cells, in part, from the assumption that lymphocytes of CML patients could be generally suppressed by the disease and/or the chemotherapy the patients receive.

In fact, in different types of leukemia, highly abnormal immune status was shown, even years after treatment discontinuation (12).

In this study, 56 CML patients in chronic phase were studied, with respect to their overall immune status and regarding the ability to recognize a bcr/abl peptide, which was previously shown to be immunogenic in normal donors.

### MATERIALS AND METHODS

**Patients.** PBMCs were obtained by venipuncture from the peripheral blood of 56 CML patients in chronic phase and from normal donors, after informed consent. PBMCs were recovered from Ficoll gradient and resuspended in RPMI 1640 plus 10% HS. CD4-enriched populations were isolated using immunomagnetic beads coated with anti-CD8 and CD4 antibodies (Dynabeads M-450 CD4; Dynal). The purified populations were subsequently checked by immunofluorescence; purified preparations contained ≥80% of CD4.

**HLA Typing.** Class II (DR and DQ loci) typing was obtained through genomic PCR with biotinilated primers, using the single specific primers hybridization assay (Inno-LiPA DRB assay; Innogenetics NV, Ghent, Belgium).

**Determiniation of the BCR/ABL Fusion Type.** A reverse transcription-PCR technique was used, as described previously (13). A single set of primers directed against the b2 exon of BCR and the a2 exon of ABL was used. The b3/a2 product generates a 417-bp band, the b2/a2 translocation produced a 342-bp band.

**PHA and TT Assay.** Purified CD4/CD8 cells, obtained from patients or donors, were seeded at 10⁵ cells/well. PHA (Murex, Temple Hile, England) was added at 1 µg/ml, and proliferation was assessed as [³H]Tdr uptake (6-h incubation) after 72 h of culture. For proliferation to TT (Connaugh Laboratory, Toronto, Canada; 10 µg/ml), 2 × 10⁵ cells/well were cultured for 6 days and labeled with [³H]Tdr for the last 18 h of culture, as described (12). Stimulation index was calculated as the ratio between mean cpm of stimulated lymphocytes and unstimulated ones.

**Delayed Type Skin Reaction to Ubiquitous Antigens.** The Multitest assay (Institut Mérieux, Lyon, France) was used. The skin was prepared with ethyl ether; the eight-prong comb containing seven different antigens (tetanus and diphtheria toxoids, mumps, candida, tricophyton, tuberculin, and streptococcus) and a negative control was then applied to the skin. Each individual skin area was allowed to absorb the overlying antigen for 2 min, and then separately wiped. The reaction was read after 48 h, as mean diameter of induration, using the ball-point technique (14).

**Peptides.** The following peptides were used: B/A1, a 17-mer Bcr/abl peptide (ATGFKQSSKALQRPVAS), was produced under Good Medical Practice conditions by Bachem AG (Bubendorf, Switzerland) and kindly supplied by Dr. O. Leeksma (Leiden University Medical Center, Leiden, the Netherlands). Peptide 12 (NSVDDLINSTKIYSYFPSVI; produced by Medprobe, Oslo, Norway) is a TT-derived peptide thaw that was shown to be presented by multiple HLA DR molecules, including DR4 (15).

**Preparation of APCs.** DCs were used as APCs. Autologous DCs were obtained from adherent PBMCs after 8–14 days of culture in RPMI 10% HS supplemented with granulocyte macrophage colony-stimulating factor (Sandoz, 200 units/ml) and interleukin-4 (kindly provided by Schering-Plough; 200 units/ml), washed, pulsed with the appropriate peptide at 50 µM at 37°C (in the presence of 2.5 µg/ml B2-m) for 4 h in serum-free RPMI (SF-RPMI), and irradiated at 4000 cGy.

**In Vitro Lymphocyte Stimulation.** Purified CD4 cells (10⁵) were stimulated with irradiated peptide-pulsed DCs (10⁵)
Cells were seeded in 24-well plates; after 4 h, HS (10%) was added. On day 10, and weekly thereafter, the cells were restimulated with equal numbers of autologous irradiated (4000 cGy) peptide-pulsed PBMCs. One day after each stimulation, interleukin-2 (10 units/ml) was added. The proliferative activity was assessed after the third stimulation and then weekly, 2 days after each stimulation.

**Proliferative Assay.** Cells (5 × 10⁴) were cultured for 48 h with irradiated (4000 cGy) autologous PBMCs in absence or presence of peptide. After an additional 18 h, cells were harvested and transferred to a filter (spot-on filtermat; Pharma- cia, Buckinghamsire, United Kingdom). [³H]ThD uptake was determined by a 1205 betaplate liquid scintillation counter (Wallac, Turku, Finland). Six replicates were performed for each experimental point. Values are expressed as mean cpm; bars represent SE and are displayed only when they exceed 5% of the respective mean.

**Statistical Analysis.** Student’s t test and Fisher’s exact test were performed. Ps < 0.05 were considered significant.

**RESULTS**

The characteristics of the patients studied are presented in Table 1. Twenty-two patients were studied at diagnosis, 14 each were treated with IFN-α or with hydroxyurea. All patients had an Eastern Cooperative Oncology Group performance status of 0-1.

The immunological competence of the patients was evaluated and is presented in Table 2. With the exception of patients in group IV, who received more intensive chemotherapy, the patient population showed a general immunocompetence, as evaluated in both PHA/Tt assays, and using skin reactions to ubiquitous antigens. When data were broken according to length of treatment and time from diagnosis, a trend in favor of patients receiving an IFN-containing regimen was present. Because most patients studied were immunological competent, those individuals presenting a b3/a2 type of fusion and a DR 0401 phenotype were selected for further studies. Eight such patients (three from groups I and
II and two from group III) were identified. Purified CD4 lymphocytes were stimulated in vitro with peptide B/A1 presented by autologous DCs. A minimum of four rounds of stimulation were performed. The results obtained in eight patients and in eight donors are presented in Fig. 1. Seven of eight donors developed specific proliferative responses (stimulation index, >2) to peptide B/A1; responses usually developed after the third or fourth stimulation cycle. All positive individuals responded at a 1-μM peptide; the increase of peptide concentration to 20 μM produced a dose-dependent increase in lymphocyte proliferation. In contrast, none of the patients studied did so, even after additional stimulation cycles. This difference between donors and patients is statistically significant (P = 0.02). It has been described that DCs obtained from CML patient express the BCR/ABL gene product and can induce antileukemic cytotoxicity (16); however, patients’ lymphocytes failed to respond to both pulsed and unpulsed autologous DCs. Both patients (with the exception of patient 3) and donors developed a positive response to an ubiquitous antigen like the TT peptide; the data obtained in the eight patients and donors studied here are presented in Fig. 1 (last column of each patient and donor); responses were generally detected in both patients and donors after the second or third stimulation cycle.

It is concluded that CML patients expressing the b3/a2 translocation and the HLA DR0401 allele were incapable to recognize a bcr/abl peptide, at difference from normal donors. These results cannot be explained by a generalized immunosuppression present in these patients.

**DISCUSSION**

Bcr/abl represents the best known example of an OFP. It is known that peptides originating from the junctional region of OFPs do represent potential new antigens. Indeed, a number of reports indicated that bcr/abl peptides can be specifically recognized by lymphocytes in both a HLA class I- and II-restricted fashion. However, all reports focused, with few exceptions, on responses obtained using donor lymphocytes as the source of antileukemic effectors.

In our study, the generation of lymphocytes specific for a bcr/abl peptide in CML patients was investigated. An important preliminary information was represented by the assessment of the immunological competence of these patients. In fact, several reports indicated that the presence of generalized immunosuppression in several types of cancers (12, 17). Our results indicate that, with the exception of few patients receiving more intensive chemotherapy, the CML patients in chronic phase analyzed in our study were immunologically competent. Although IFN-receiving patients (group II) produced in some cases higher responses than patients in groups I or III, no group substantially differed from the normal donor group (V).

Our study was focused on a peptide derived from the b3/a2 type of bcr/abl protein that was previously shown to be immunogenic for donor lymphocytes expressing the HLA DR0401 molecule and to cross-react with leukemic blasts expressing the HLA DR0401 specificity and the b3/a2 type of BCR/ABL fusion.

To obtain a sufficient number of such patients (b3/a2 and HLA DR-401), a large population had to be screened. Our results indicate that patients’ lymphocytes, although functionally competent and expressing both the right type of fusion protein and the appropriate restriction element, failed to recognize peptide B/A1.

Because no generalized immunosuppression was found in these patients, tolerance to peptide B/A1 should be present. In addition, because DCs obtained from the patients’ lymphocytes could effectively present a TT peptide, the observed lack of response to peptide B/A1 seems to be more linked to a T-cell defect than to an APC defect. Our data do not permit to discriminate whether such tolerance was induced by the leukemic cells or whether it preceded the onset of leukemia. It is worth noting that some distinct HLA specificities are associated with a decreased incidence of CML (18); therefore, it is possible that some HLA molecules be more (or less) capable to present peptides derived from the bcr/abl protein. However, no such information is available for HLA DR0401.

On the other hand, our data, as well as previously published reports (7), show that HLA DR0401 can, indeed, present peptide B/A1, either in a synthetic form or derived through the intracellular processing of the bcr/abl protein in leukemic cells.

Thus, the most likely explanation for our results can reside in the induction of tolerance operated by the leukemic cells in the patients studied and reported here. It is worth mentioning that these patients, although in stable chronic phase, were all PCR positive and harbored a significant tumor load of several billion leukemic cells. It is, therefore, possible that the high number of leukemic cells cause tolerance to the bcr/abl protein by anergyizing specific lymphocytes.

These data suggest that strategies aimed at bypassing such tolerance should be the object of future research. In addition, it is possible that different peptides deriving from the junctional region of the bcr/abl protein could result more immunogenic in CML patients. However, as they presently stand, these results do not support the use of peptide B/A1 in immunotherapeutic approaches to CML. Alternative strategies, either immunological or nonimmunological (19, 20), are needed to achieve specific control of CML and of the other neoplasias caused by the bcr/abl OFP.

**REFERENCES**


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