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OLIGONUCLEOTIDE IMT504 INDUCES AN IMMUNOGENIC PHENOTYPE AND APOPTOSIS IN CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Abtract Oligonucleotides (ODNs) of the PyNTTTTGT class directly stimulate B lymphocytes and plasmacytoid dendritic cells of the immune system of primates. Here we investigated the ability of the PyNTTTTGT ODN prototype IMT504 to regulate the expression of surface molecules and apoptosis in human B-chronic lymphocytic leukemia (CLL) cells. The surface molecules CD25, CD40, CD80 and CD86 were up-regulated upon incubation of the B-CLL cells with IMT504. Co-stimulation with IL-2 resulted in further up-regulation. IMT504-activated B-CLL cells were also good stimulators of T cells in allogeneic mixed lymphocyte reactions and co-stimulation with IL-2 improved this stimulation capacity. Apoptosis of the B-CLL cells *in vitro* was also stimulated by incubation with IMT504. In this case, co-stimulation with IL-2 was not significant. Furthermore, B-CLL cells of all the patients studied developed an immunogenic phenotype and entered stimulated apoptosis upon *in vitro* incubation with IMT504 independently of the mutational status of their IgV_H genes, becoming a good marker for tumor progression.

Key words: chronic lymphocytic leukemia, immunostimulatory ODN, immunogenic phenotype, IL-2, V_H genes mutation

Resumen ***El oligonucleótido IMT504 induce un fenotipo immunogénico y apoptosis en células de leucemia linfocítica crónica.*** Los oligonucleótidos (ODNs) de tipo PyNTTTTGT estimulan directamente las células B y las células dendríticas plasmacitoides del sistema inmune de primates. En este trabajo, investigamos la habilidad del IMT504, prototipo de los ODN tipo PyNTTTTGT, para regular la expresión de moléculas de superficie y la apoptosis en células B de leucemia linfocítica crónica (LLC). La expresión de las moléculas de superficie CD25, CD40, CD80 y CD86 fue aumentada al incubar las células B-LLC con IMT504. La co-estimulación con IL-2 provocó un aumento mayor. Las células B-LLC activadas fueron buenas estimuladoras de las células T en cultivo mixto de linfocitos alogeneicos y la co-estimulación con IL-2 mejoró esta capacidad. La apoptosis de las células B-LLC también fue estimulada por incubación con IMT504. En este caso, la co-estimulación con IL-2 no fue significativa. Más aún, las células B-LLC de todos los pacientes estudiados, desarrollaron un fenotipo inmunogénico y entraron en apoptosis luego de la incubación *in vitro* con IMT504, independientemente del estado mutacional de sus genes IgV_H, un indicador del pronóstico de la patología.

Palabras clave: leucemia linfocítica crónica, ODN inmunoestimulante, fenotipo inmunogénico, IL-2, mutación genes V_H

Chronic lymphocytic leukemia (CLL) is a clonal malignancy that results from expansion of the mature lymphocyte compartment. CLL cells are slowly dividing monoclonal B cells, generally of the CD5+ B cell lineage, which express major histocompatibility complex (MHC) class I and II antigens and surface immunoglobulin (Ig). The clinical course of CLL is heterogeneous, with some patients progressing rapidly and others exhibiting a more stable disease that can last for many years. The mutation status of the immunoglobulin V_H genes expressed in CLL cells is a powerful prognostic marker and patients with V_H gene mutations have a clearly better prognosis than patients with unmutated (or quasi unmutated) V_H genes[1, 2].

CLL cells have many genetic features that distinguish them from normal B cells. These genetic differences may result in the expression of tumor antigens that if recognized by the immune system should result in a cytotoxic T-cell antitumoral answer. However, although B-CLL pa-

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tients have normal functional T cells[3], B-CLL cells are not recognized by the immune system. This seems to be related to the fact that they lack several co-stimulatory molecules necessary for an efficient T-cell activation[4, 5]. In agreement with this, it has been reported that immunogenicity of B-CLL cells can be enhanced by up regulation of costimulatory molecules via the CD40-CD40 ligand (L) pathway[6,7]. In regard to this, encouraging results have been reported in a phase I clinical trial based on the infusion of autologous leukemia cells transduced with a viral vector for expression of CD40-L[8]. An immunocompetent phenotype could also be induce in B-CLL cells by incubation either with immunostimulatory CpG oligonucleotides (CpG-ODNs)[9, 10] or with CpG-ODNs plus interleukin 2 (IL-2)[11]. These oligonucleotides can directly activate B cells and plasmacytoid dendritic cells[12-16] and are potent adjuvants, promoting cellular and humoral immune responses specific for a variety of antigens[17-24]. CpG-ODNs have also been proved to be effective in animal models of cancer and allergy[25-28] and several clinical trials using CpG ODNs are currently in progress[29]. Recently, a new class of potent immunostimulatory ODNs with similar properties, the PyNTTTTGT ODNs, has been described[30]. These ODNs are mainly active in humans and other primates and their prototype is the so-called IMT504. Considering the above-mentioned information, we investigated whether an immunogenic phenotype could be induced in B-CLL cells by incubation with this novel ODN IMT504 either alone or in combination with IL-2 and whether the induction depend upon the prognosis of the CLL evaluated by the mutation status of the V_H genes of each patient. Furthermore, we evaluated the ability of the ODN-IMT504 to modify the apoptosis of the CLL cells *in vitro*.

Materials and Methods

Patients

The study comprised blood samples from 20 patients with B-CLL diagnosed according to established morphologic and immunophenotypical criteria[31, 32]. Samples were obtained after informed consent. The age of patients ranged from 48 to 77 (median, 63). The sex ratio (male to female) was 1.5. Half of the patients had undergone chemotherapy before the study. Staging information was as follows: eight were Rai stage 0, four stage 1, five stage 2, one stage 3 and two stage 4. Leukocyte counts ranged from 10 000 to 105 000 (median 48 900).

Oligodeoxynucleotides (ODNs)

Desalted phosphorothioate (S) ODNs were purchased from Oligos ETC (Bethel, Maine). ODNs were suspended in depyrogenated water and kept at -20 °C until used. The following phosphorothioate ODNs were used: IMT 504 (TCATCATTTTGTCATTTTGTCATT) as immunostimulatory ODN and IMT022 (TGCTGCAAAAGAGCAAAAGAGCAA) as a negative control. ODNs were used at a final concentration of 1.5 µg/ml.

Interleukin 2

Human recombinant IL-2 was kindly provided by Laboratorio Pablo Cassará (Buenos Aires, Argentina). IL-2 was used at a final concentration of 400 IU/ml.

Peripheral Blood Mononuclear Cells (PBMC)

Blood samples were obtained by venipuncture from CLL patients of the hematological division of the CEMIC (*Centro Médico de Investigaciones Clínicas*, Buenos Aires, Argentina) using heparin as anticoagulant. PBMC were isolated by Ficoll-Hypaque (Sigma Diagnostics Inc., St. Louis, MO) density gradient centrifugation. Briefly, blood samples diluted 1:2 in RPMI-1640 medium (PAA laboratories GmbH, Linz, Austria) supplemented with 2.0 mM L-glutamine and 50.0 µg/ml gentamicin and 20 mM HEPES, were centrifuged at 1000 × g for 40 min at 20 °C. PBMC were isolated, washed and suspended in medium supplemented with 10% fetal calf serum.

Purification of cells

B-lymphocytes and T-lymphocytes were purified from PBMC using magnetic cell separation systems (MACS) (Miltenyi Biotec, Germany). B cells were positively isolated using micro beads conjugated to antibodies against human CD19. T cells were positively isolated using micro beads conjugated to antibodies against human CD4. Cell purity was more than 96% according to flow cytometric assays.

Mixed lymphocyte reaction

Purified B-CLL cells were plated at 37 °C in a 5% CO_2 humidified atmosphere for 48 h a) either with or without ODN, b) either with or without IL-2 or c) with both ODN and IL-2. After this, the cells were washed twice, incubated for 30 min with 50 µg/ml mitomycin C (Sigma Chemical Co., St. Louis, MO) and washed four times with 11 ml of RMPI-1640. 1×10^5 of these mitomycin-inactivated B-CLL cells were co-cultured in 96-well microtiter plates with 5×10^4 purified T cells per well (stimulator/responder ratio 2:1) and incubated for five days. For the last 16 hours of culture, the mix was pulsed with 1 µCi of ^{3}H-Thymidine (Amersham Biosciences, Specific activity 22.0 Ci/mmol). Cells were harvested onto glass-fiber filters and ^{3}H thymidine incorporation measured by scintillation counting. Standard deviations of quadruplicate wells were less than 10%.

Flow cytometry

Staining of surface antigens was performed as described[33]. Anti CD19 (Clone LT19), CD5(Clone CD5-5D7), CD20 (Clone 2H7), CD25 (Clone MEM-181), CD40 (clone LOB 7/6), CD80 (Clon MEM-233) and CD86 (Clone BU63 antibodies were purchased from Serotec (Raleigh, NC, USA). Flow cytometric data of 10 000 cells/sample were acquired on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). Data were analyzed using the computer program Win MDI 2.8, Interface Flow Cytometry Application (Joseph Trotter Copyright 1993-1998).

Apoptosis

Apoptosis was evaluated using an Annexin V combined with Propidium Iodide (PI) staining kit (Serotec, Oxford, UK) following manufacturer's guidelines. Briefly, 2×10^5 B-CLL cells were washed with cold PBS and suspended in 195 µl of annexin-binding buffer plus 5 µl of FITC-labeled annexin V. After 15 min incubation at room temperature in the dark, cells were washed with cold PBS and suspended in 190 µl of bind-

ing buffer plus 10 µl of PI. The stained cells were immediately analyzed by flow cytometry.

Preparation of RNA and cDNA synthesis

Total RNA was isolated from PBMC using TRIZOL Reagent® (Invitrogen™ life technologies, Carlsbad, CA 92008 USA) according to the manufacturer's instructions. One or two µg of RNA were reverse-transcribed to cDNA using 200U of Superscript™ II RNAase H-Reverse Transcriptase (Invitro-gen™ life technologies), 40U of RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen™ life technologies) and 1 µl of oligo $dT_{12\text{-}18}$ primer (500µg/ml) (Invitrogen™ life technologies) in a total volume of 20 µl. Reactions were carried out at 42 °C for 50 min and heated at 70 °C for 15 min to stop the reactions.

PCR conditions for V_H family assignment

In order to determine the V_H gene family used by each B-CLL cells, 1-2 µl of cDNA were amplified using a mixture of sense primers specific for each of the leader sequences of the V_H families: VH1 CTCACCATGGACTGGACCTGGAG; VH2 ATGGACATACTTTGTTCCACGCTC; VH3 CATGGAGTTTGGGCTGAGCTGG; VH4 ACATGAAACAYCTGTGGTTCTTCC; VH5 ATGGGGTCAACCGCCATCCTCCG; VH6 ATGTCTGTCTCCTTCCTCATCTTC and an appropriate antisense C_H primer: IgM: CAGGAGAAAGTGATGGAGTCG; IgG: GGGGAAGT-AGTCCTTGACCAG; IgA: GAGGCTCAGCGGGAAGACCTT. Reactions were carried out in 50 µl using 25 pmol of each primer.

Cycling was performed with a Mastercycler 5333 (Eppendorf AG, Hamburg) as follows: denaturation at 94 °C for 45 s; annealing at 55 °C for 30 s; and extension at 72 °C for 45 s. After 35 cycles, extension was continued for an additional 10 min-period.

Sequencing of PCR products

PCR products were sequenced after purification with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway NJ 08855 USA), using the CEQTMDTCS-Quick Start Kit (Beckman Coulter, USA) and an automated sequencer (CEQ 2000, Beckman Coulter). At least 3 replicates were run for each PCR product.

Analyses of V_H, D, and J_H sequences

Sequences were compared with those in the V BASE sequence directory[34] using the Vector NTI 6 software (Invitrogen, California). In those instances where a deviation of more than 1% from the germline sequence was found, the algorithm of Chang and Casali[35] was used to determine whether antigen selection of the replacement (R) mutations had occurred. The expected number of R mutations in CDR was calculated using the formula $R = n \times CDR\ Rf \times CDRrel$, where n is the total number of observed mutations, Rf is the replacement frequency inherent to the CDR, and CDRrel is the relative size of the segment. A binomial probability (p) model was used to evaluate whether the excess of R mutations in CDR was due to chance[35].

Results

Stimulation of the expression of the IL-2 receptor (CD25) on B-CLL cells in response to IMT504

It has been reported that ODNs of the class CpG are able to induce the expression of IL-2 receptor on B-CLL cells[12]. Therefore it was of interest to evaluate if ODNs of the new class PyNTTTTGT were also able to induce this same receptor. Purified B-CLL cells and normal peripheral blood B cells derived from healthy donors were incubated with the immunostimulatory ODN PyNTTTTGT prototype IMT504 or the negative control ODN IMT022[30]. After a 48 h incubation period, CD25 expression was evaluated by FACS analysis (Fig. 1). As can be observed, IMT504 was able to up regulate the expression CD25 on B-CLL cells (Fig. 1B). However, stimulation of normal cells was very poor or null (Fig. 1A).

Regulation of the expression of CD25, CD40, CD80 and CD86 on B-CLL cells by IMT504 alone or in combination with IL-2

B-CLL cells are usually poor antigen-presenting cells because they have low levels of co-stimulatory molecules necessary for T-cell activation[4, 5]. On the other hand, as shown above, incubation with IMT504 increases the amount of the IL-2 receptor. Thus, it was important to investigate if incubation of the B-CLL cells with IMT504 resulted in induction of co-stimulatory molecules and if there was any cooperation between IMT504 and IL-2 in the induction of CD25 and co-stimulatory molecules. Fig. 2 shows a typical result of a FACS analysis after incubation of the B-CLL cells with IMT504 or the negative control ODN IMT022.

As can be observed, IMT504 was able to up regulate the expression of all these surface molecules. In contrast, the ODN control IMT022 was inactive. Furthermore, synergy with IL-2 was observed in all cases. Results were qualitatively similar for B-CLL cells extracted from each of the 20 patients analyzed. However, stimulation by IMT504 or IMT504+IL-2 was highly variable with fluorescence indexes fluctuating between 4 and 20 for CD25, 3 and 20 for CD40, 1.4 and 3 for CD80 and 1.5 and 4 for CD86. Differences in the fluorescence index between B-CLL cells incubated either with or without IMT504 were always statistically significant for CD25 and CD40. However, these differences were not statistically significant for the CD80 and CD86 surface molecules in about 30% of the samples. These results indicate that B-CLL cells can acquire a potentially immunogenic phenotype by incubation with IMT 504 or even better with IMT504+IL-2.

On the other hand, it was observed that expression of CD20, a surface antigen expressed primarily on B cells, was either not modified or slightly diminished on B-CLL cells upon incubation with ODN IMT504 or ODN 2006 (an ODN widely used as human CpG type B prototype)[29] (not shown). This is in contrast with a previous report indicating that CD20 is up regulated in B-CLL in response to the CpG ODN 2006[10].

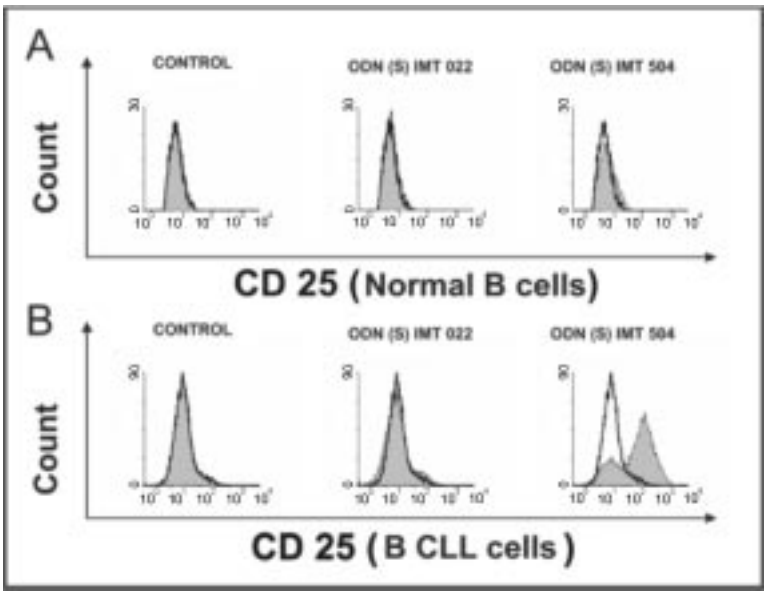

Fig. 1.– Induction of the IL2 receptor (CD25) on B-CLL cells by IMT504 Purified B-CLL cells were cultured for 48 h with the immunostimulatory oligonucleotide IMT504 or the negative control oligonucleotide IMT022 and then stained with fluorescent anti-CD19, anti-CD5 and anti-CD25 antibodies. Flow cytometric results are presented as histograms corresponding to cells in the CD19, CD5 double positive gate for B-CLL cells and in the CD19 positive gate for normal B-cells.

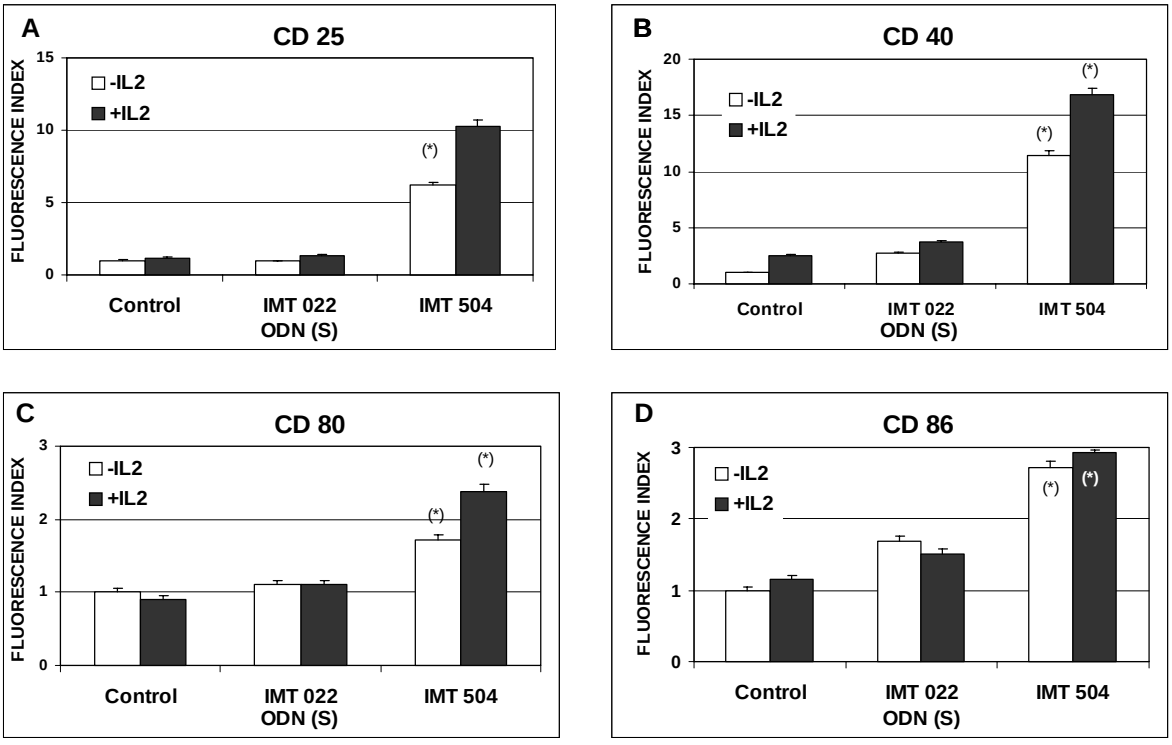

Fig. 2.– Induction of CD25, CD40, CD80 and CD86 on B-CLL cells by IMT504 or IMT504+IL2. B-CLL cells were cultured for 48 h either with or without IL2, IMT504, IMT504+IL2, IMT022 or IMT022+IL2 and then stained with fluorescence anti-CD19, anti-CD5 and anti-CD25 (A), anti-CD40 (B), anti-CD80 (C) or anti-CD86 (D). Flow cytometric results are presented as the mean fluorescence of cells in the CD19, CD5 double positive gate, relative to the mean fluorescence of cells incubated without ODN (fluorescence index).

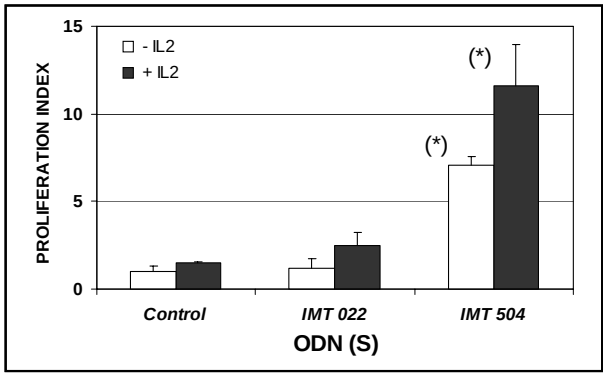

Fig. 3. Activation of allogeneic T cells by B-CLL cells incubated with IMT504 or IMT504+IL2. B-CLL cells were cultured in the presence or absence of IL2, IMT504, IMT504+IL2, IMT022 or IMT022+IL2 for 48 h. After this, B-CLL cells were washed, inactivated with mitomycin C and co-cultured with purified allogeneic T cells. After 5 days cells were pulsed with ^{3}H-Thymidine. Data represent the mean proliferation index and standard deviation of quadruplicate assays. Statistical significance was evaluated by Student's *t* test. *, Statistically significant differences ($p < 0.05$) compared with controls.

Activation of allogeneic T cells by B-CLL cells incubated with IMT504 or IMT504+IL-2

B-CLL cells are poor stimulators of T cells in allogeneic mixed lymphocyte reactions. However, it has been reported that if the expression on the surface of the B-CLL cells of co-stimulatory molecules is up-regulated these cells are able to induce allogeneic immune recognition[11, 12, 36].

The capacity for allogeneic T-cell activation of B-CLL cells incubated with IMT504, IL-2 or both was evaluated in mixed lymphocyte reactions. Fig. 3 shows that while no increase of the proliferation index was observed when B-CLL cells were incubated with IL-2 alone, incubation with IMT504 resulted in an enhancing of the proliferation index. On the other hand, the enhancing effect was significantly larger if the B-CLL cells were incubated with IMT504+IL-2. Stimulation by incubation with IMT504 or IMT504+IL-2 was observed in all the three cases studied. However, the magnitude of the stimulation was highly variable with stimulation indexes of about 2 to 7 for cells incubated with IMT504 and of 3 to 12 for cells incubated with IMT504+IL-2. Variation may be owed to the B-CLL cells, to the T cells or to both. These results reinforce the idea that B-CLL cells acquire an immunogenic phenotype after incubation with IMT504 and that further enhancement could be achieve by combination of IMT504 and IL-2.

Effect of IMT504 on the apoptosis of the CLL cells in vitro

B-CLL cells have defects in apoptotic pathways and therefore accumulate *in vivo*. However, when removed from the patient and cultured *in vitro*, these malignant cells rapidly undergo apoptosis[37,38]. It has also been reported that CpG immunostimulatory oligonucleotides can modify the *in vitro* apoptosis of the B-CLL cells in an apparent complex fashion. The apoptosis of B-CLL cells from patients with high serum thymidine kinase activity is inhibited, while apoptosis of B-CLL cells from patients with low thymidine kinase activity is stimulated[10]. Taking into account this information we decided to investigate the effect of IMT504 on the apoptosis of B-CLL cells *in vitro*. Fig. 4 resumes the results. Line A shows a typical result for normal B cells. After 40 h incubation *in vitro* more than 60% of the cells remain intact (lower left case), between 15% and 20% had entered apoptosis (lower right case) and about the same number of cells had entered necrosis (upper right case). Incubation of these normal cells with ODN IMT504 clearly protected them from necrosis while incubation with the negative control ODN IMT022 had no effect. Line B shows a typical result observed in 60% of the B-CLL cells studied (subgroup 1). These cells were more prone to enter necrosis, presumptively through a rapid passage through the apoptotic stage. Only between 10% and 40% remained intact after 40 h. incubation *in vitro*. Incubation of these B-CLL cells with ODN IMT504 clearly induced apoptosis but protected them from necrosis. Incubation with the negative control ODN IMT022 has not effect. Line C shows a typical result observed in the remaining (40%) of the B-CLL cells studied (subgroup 2). These cells had a low tendency to enter apoptosis or necrosis *in vitro* like normal B cells. After 40 h incubation with ODN IMT504 there was a clear tendency of these B-CLL cells to enter apoptosis but necrosis augmented or remained unchanged. Incubation with the negative control ODN IMT022 had no effect. Co-stimulation with IL-2 was, in all these cases, not effective (not shown).

Mutational status of the Ig V_H genes of the B-cells studied

Unmutated Ig V_H genes are associated with a more aggressive form of CLL[1, 2]. Therefore, it was of interest to evaluate if B-CLL cells of patients with poor prognosis were able to respond to the ODN IMT504 immunostimulation. The mutational status of the Ig V_H genes of 12 patients out of the 20 analyzed for induction of co-stimulatory molecules in their B-CLL cells was investigated. Table 1 show that seven of these patients had CLL cells with unmutated Ig V_H genes. The average response of these patients, measured by the mean fluorescence intensity stimulation index of the CD40 marker was 4.68 (SD: 2.61) not significantly different, according to the Student test, from the average response corresponding to the five patients with mutated Ig V_H genes that was 6.92 (SD: 3.89). Similar results were obtained using the mean fluorescence

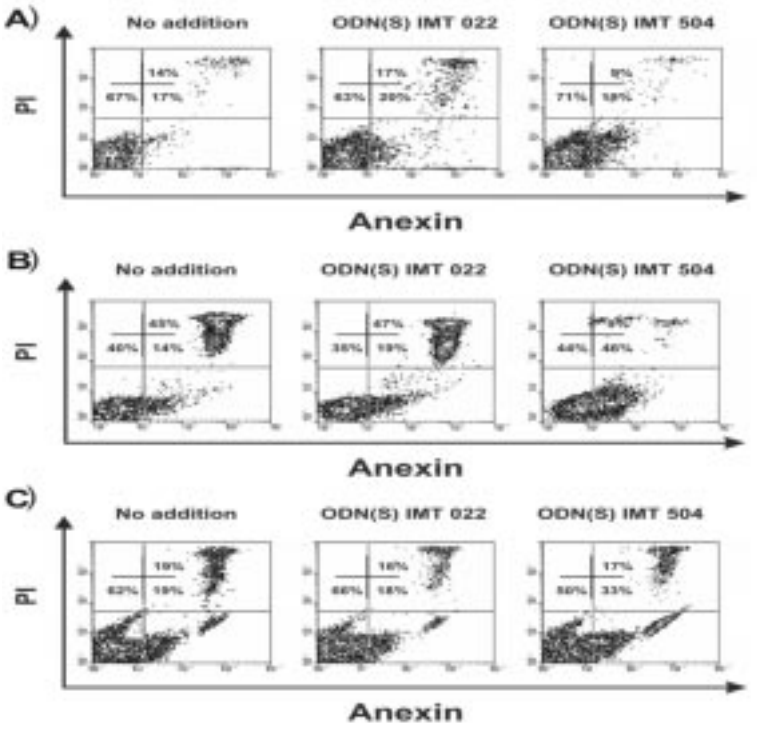

Fig. 4.– Induction of apoptosis in B-CLL cells incubated with IMT504. B-CLL cells were incubated in the presence or absence of IMT504 or IMT022 for 40 h. After this, cells were treated with FITC-labeled annexin V and Propidium Iodine (PI) and analyzed by flow cytometry. The lower left box represents the non-apoptotic cells, the lower right box the apoptotic ones and the upper right box the secondary necrotic ones. (A): normal B-cells, (B): B-CLL cells, subgroup 1, (C): B-CLL cells, subgroup 2.

intensity stimulation index of CD80 and CD86 (not shown). On the other hand, apoptosis of B-CLL cells of all the patients here studied was stimulated by incubation with IMT504 and there was no correlation between the mutation status and the apoptosis subgroup (1 or 2). Therefore, these results indicate that the biologic effects that IMT504 has on the B-CLL cells of a given patient, are independent of the prognostic of this patient as evaluated by the mutational status of the Ig V_H genes

Discussion

In this study we investigated the potential of the immunostimulatory PyNTTTTGT prototype ODN IMT504 and of IMT504+IL-2 to induce co-stimulatory molecules expression and apoptosis in B-CLL cells. It was also investigated if the effects attributable to IMT504 were in some way related to the prognosis of the CLL as predicted by the mutational status of its Ig VH genes.

IMT504 is an ODN containing two immunostimulatory motifs of the novel PyNTTTTGT class[30]. ODNs containing motifs of this class directly stimulate normal B cells inducing secretion of cytokines (e.g. IL6), immunoglobulins (e.g. IgM) and expression of co-stimulatory molecules (e.g. CD40). In this study, it was found that IMT504 was able to induce the expression of CD40, CD80 and CD86 co-stimulatory molecules on the surface of B-CLL cells. Furthermore, it was found that the combination of IMT504 with IL-2 was more effective than any of these individual molecules. This is important in order to induce tumor-reactive T cells that could mediate tumor regression. For example, stimulation of an immunogenic phenotype, via interaction of CD40/CD40L, has been used, with promising results, in clinical trials for therapy of CLL[8]. At first glance, it seems that a therapy based on pharmacologically active molecule such as an ODN would be more convenient than a therapy that involves gene transfection in each case. Regarding this, it can be said that our pre-clinical trials (unpublished data) have demonstrated that IMT504 is a drug with very low toxicity.

It was also demonstrated that ODN IMT504 is able to induce apoptosis of the B-CLL cells *in vitro*. This is significant since, in general, immunoestimulatory ODNs have a tendency to rescue normal B cells from spontaneous apoptosis[39,40]. We observed that ODN IMT504 protected

TABLE 1.– *Sequence homology of the immunoglobulin heavy chain variable region (V_H) of patients with the nearest germline V_H gene*

Patient ID	RAI stage	Nearest V_H gene and locus	Direct identity (%)	Chang-Casali *Correction* CDR *p*	Mutational status	CD 40 stimulation index
1	0	V_H3 8-1B/3-66	99.3	-	Unmutated	7.48
2	1	V_H4 DP-71/3d197d/4-59	99.7	-	Unmutated	5.46
3	2	V_H3 YAC-9/COS-27/3-73	97.7	$8.204X10^{-1}$	Unmutated	8.45
4	2	V_H3 8-1B/3-66	99.3	-	Unmutated	4.34
5	4	V_H4 VIV-4/4.35/4-04	96.7	$3.462X10^{-1}$	Unmutated	1.44
6	0	V_H4 DP-63/VH4.21/4-34	97.6	$2.819X10^{-1}$	Unmutated	3.36
7	0-1	V_H3 DP-29/12-2/3-72	96.7	$3X10^{-1}$	Unmutated	2.26
8	1	V_H4 DP-63/VH4.21/4-34	93.5	$3.47X10^{-2}$	Mutated	6.85
9	0	V_H3 DP-49/1.9III/3-30/3-30.5	93.9	$5X10^{-4}$	Mutated	4.60
10	0	V_H1 DP-8/1-02	96.9	$5.8X10^{-3}$	Mutated	9.27
11	2	V_H3 LSG 6.1/-	97.2	$3.32X10^{-2}$	Mutated	1.96
12	na	V_H4 DP-63/VH4.21 /4-34	93.1	$4.6X10^{-3}$	Mutated	11.93

normal B cells, from the spontaneous necrosis observed when these cells were incubated in the absence of the ODN. However, the number of apoptotic cells was not significantly modified by this ODN. In contrast, the number of apoptotic cells was significantly increased by incubation with IMT504 in all the experiments where B-CLL cells were used instead of normal cells. In many, but not all, of these experiments protection against necrosis, as in normal B-cells was also observed. These results suggest that there are at least two different apoptotic pathways acting on B-CLL cells during our *in vitro* assays. One of them would be responsible for the high spontaneous apoptosis commonly observed in these cells *in vitro* (a process that results in a rapid passage to the necrotic stage); and another one induced by IMT504, (a process that results in the accumulation of more stable apoptotic cells). Spontaneous apoptosis of B-CLL is not likely to be an important mechanism operative *in vivo*. On the contrary, it is well known that B-CLL cells are very resistant to natural apoptosis *in vivo* Therefore, induction of apoptosis in B-CLL cells by IMT504, which may well be operative *in vivo*, could be considered another fact predicting a good performance of this ODN in a potentially applicable immunotherapy of B-CLL.

Recently, it has been demonstrated that the mutational status of the Ig heavy-chain variable-region (IgV_H) genes in the B-CLL cells is an important prognostic factor in the disease[1,2]. Patients with IgV_H gene mutations have a better prognosis than patients with unmutated (or quasi unmutated) IgV_H genes. The study of the mutational status of the IgV_H genes of B-CLL cells of the patients here studied indicated that about 60% have unmutated genes and 40% have mutated IgV_H ones. However, B-CLL cells from all these patients develop an immunogenic phenotype and entered apoptosis upon incubation with ODN IMT504 in a similar fashion. Therefore, it could be predicted that an immunotherapy of B-CLL using ODN IMT504, if successful, may benefit both patients with good and poor prognosis.

In summary, the present results demonstrate that IMT504, the prototype of the immunostimulatory ODNs of the PyNTTTTGT class, is able to induce in B-CLL cells an immunogenic phenotype and apoptosis independently of the prognostic of the leukemia resulting of the analysis of the mutational status of the Ig V_H genes. Therefore, IMT504 alone or in combination with other anti-leukemic treatment may contribute to the treatment of B-CLL leukemia and clinical trials to investigate this are warranted.

References

1. Fais F, Ghiotto F, Hashimoto S, et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J Clin Invest* 1998; 102: 1515-25.
2. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* 1999; 94: 1848-54.
3. Decker T, Flohr T, Trautmann P, et al. Role of accessory cells in cytokine production by T cells in chronic B-cell lymphocytic leukemia. *Blood* 1995; 86: 1115-23.
4. Dazzi F, D'Andrea E, Biasi G, et al. Failure of B cells of chronic lymphocytic leukemia in presenting soluble and alloantigens. *Clin Immunol Immunopathol* 1995; 75: 26-32.
5. Ranheim EA, Kipps TJ. Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. *J Exp Med* 1993; 177: 925-35.
6. Van den Hove LE, Van Gool SW, Vandenberghe P, et

al. CD40 triggering of chronic lymphocytic leukemia B cells results in efficient alloantigen presentation and cytotoxic T lymphocyte induction by up-regulation of CD80 and CD86 costimulatory molecules. *Leukemia*. 1997; 11: 572-80.
7. Kato K, Cantwell MJ, Sharma S, Kipps TJ. Gene transfer of CD40-ligand induces autologous immune recognition of chronic lymphocytic leukemia B cells. *J Clin Invest* 1998; 101: 1133-41.
8. Wierda WG, Cantwell MJ, Woods SJ, et al. CD40-ligand (CD154) gene therapy for chronic lymphocytic leukemia. *Blood* 2000; 96: 2917-24.
9. Decker T, Schneller F, Sparwasser T, et al. Immunostimulatory CpG-oligonucleotides cause proliferation, cytokine production, and an immunogenic phenotype in chronic lymphocytic leukemia B cells. *Blood* 2000; 95: 999-1006.
10. Jahrsdorfer B, Hartmann G, Racila E, et al. CpG DNA increases primary malignant B cell expression of costimulatory molecules and target antigens. *J Leukoc Biol* 2001; 69: 81-8.
11. Decker T, Scneller F, Kronschnabl M, et al. Immunostimulatory CpG-oligonucleotides induce functional high affinity IL-2 receptors on B-CLL cells: Costimulation with IL-2 results in a highly immunogenic phenotype. *Exp Hematol* 2000; 28: 558-68.
12. Krieg AM, Yi AK, Matson S, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995; 374: 546-9.
13. Kadowaki N, Antonenko S, Liu YJ. Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c- type 2 dendritic cell precursors and CD11c+ dendritic cells to produce type I IFN. *J Immunol* 2001; 166: 2291-5.
14. Krug A, Rothenfusser S, Hornung V, et al. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *Eur J Immunol* 2001; 31: 2154-63.
15. Krug A, Towarowski A, Britsch S, et al. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *Eur J Immunol* 2001; 31: 3026-37.
16. Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J Immunol* 2000; 164: 944-53.
17. Sparwasser T, Koch ES, Vabulas RM, et al. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur J Immunol* 1998; 28: 2045-54.
18. Ballas ZK, Rasmussen WL, Krieg AM. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J Immunol* 1996; 157: 1840-5.
19. Hornung V, Rothenfusser S, Britsch S, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* 2002; 168: 4531-7.
20. Lipford GB, Bauer M, Blank C, et al. CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *Eur J Immunol* 1997; 27: 2340-4.
21. Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J Exp Med* 1997; 186: 1623-31.
22. Moldoveanu Z, Love-Homan L, Huang WQ, Krieg AM. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. *Vaccine* 1998; 16: 1216-24.
23. Davis HL, Weeratna R, Waldschmidt TJ, et al. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J Immunol* 1998; 160: 870-6.
24. Weeratna RD, McCluskie MJ, Xu Y, Davis HL. CpG DNA induces stronger immune responses with less toxicity than other adjuvants. *Vaccine* 2000; 18: 1755-62.
25. Kline JN, Waldschmidt TJ, Businga TR, et al. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J Immunol* 1998; 160: 2555-9.
26. Magone MT, Chan CC, Beck L, Whitcup SM, Raz E. Systemic or mucosal administration of immunostimulatory DNA inhibits early and late phases of murine allergic conjunctivitis. *Eur J Immunol* 2000; 30: 1841-50.
27. Wooldridge JE, Ballas Z, Krieg AM, Weiner GJ. Immunostimulatory oligodeoxynucleotides containing CpG motifs enhance the efficacy of monoclonal antibody therapy of lymphoma. *Blood* 1997; 89: 2994-98.
28. Carpentier AF, Chen L, Maltonti F, Delattre JY. Oligodeoxynucleotides containing CpG motifs can induce rejection of a neuroblastoma in mice. *Cancer Res* 1999; 59: 5429-32.
29. Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* 2002; 20: 709-60.
30. Elias F, Flo J, Lopez RA, et al. Strong cytosine-guanosine-independent immunostimulation in humans and other primates by synthetic oligodeoxynucleotides with PyNTTTTGT motifs. *J Immunol* 2003; 171: 3697-3704.
31. Rozman C, Montserrat E. Chronic lymphocytic leukemia. *N Engl J Med* 1995; 333: 1052-7.
32. Weisenburger DD, Armitage JO. Mantle cell lymphoma-an entity comes of age. *Blood* 1996; 87: 4483-94.
33. Flo J, Massouh E. Age-related changes of naive and memory CD4 rat lymphocyte subsets in mucosal and systemic lymphoid organs. *Dev Comp Immunol* 1997; 21: 443-53.
34. Cook GP, Tomlinson IM. The human immunoglobulin VH repertoire. *Immunol Today* 1995; 16: 237-42.
35. Chang B, Casali P. The CDR1 sequences of a major proportion of human germline Ig VH genes are inherently susceptible to amino acid replacement. *Immunol Today* 1994; 15: 367-73.
36. Buhmann R, Nolte A, Westhaus D, Emmerich B, Hallek M. CD40-activated B-Cell chronic lymphocytic leukemia cells for tumor immunotherapy: stimulation of allogeneic versus autologous T cells generate different types of effector cells. *Blood* 1999; 93: 1992-2002.
37. Collins RJ, Verschuer LA, Harmon BV, et al. Spontaneous programmed death (apoptosis) of B-chronic lymphocytic leukemia cells following their culture in vitro. *Br J Haematol* 1989; 71: 343-50.
38. McConkey DJ, Aguilar-Santelises M, Hartzell P, et al. Induction of DNA fragmentation in chronic B-lymphocytic leukemia cells. *J Immunol* 1991; 146: 1072-6.
39. Yi AK, Peckham DW, Ashman RF, Krieg AM. CpG DNA rescues B cells from apoptosis by activating NFkappaB and preventing mitochondrial membrane potential disruption via a chloroquine-sensitive pathway. *Int Immunol* 1999; 11: 2015-24.
40. Wang Z, Karras JG, Colarusso TP, Foote LC, Rothstein TL. Unmethylated CpG motifs protect murine B lymphocytes against Fas-mediated apoptosis. *Cell Immunol* 1997; 180: 162-7.