Reactive arthritis after BCG immunotherapy: T cell analysis in peripheral blood and synovial fluid

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Abstract

Objective. To investigate the pathogenic mechanism of reactive arthritis after instillation of Calmette–Guérin bacillus (BCG). Although the clinical features of reactive arthritis after BCG therapy are well described, only a few reports have studied the possible pathogenic mechanisms.

Methods. We analysed by flow cytometry the phenotype and T-cell receptor (TCR) expression of peripheral blood (PB) and synovial fluid (SF) T cells in a patient who developed reactive arthritis (ReA) following intravesical BCG immunotherapy for bladder cancer. The proliferative response of short-term T-cell lines (TCL) from PB of this patient to mycobacterial antigens was tested by bromodeoxyuridine incorporation.

Results. CD4+ and CD8+ SF T cells with activated and memory phenotype were observed at the onset of arthritis. We were able to detect BV-restricted expansion of CD8+ T cells in PB (BV17) and in SF (BV5S1 and BV12). The percentage of PB and SF CD8+ T cells that expanded diminished when the symptoms remitted. The strongest response of CD4+ TCL from the patient in vitro was obtained for human hsp-60 in an inversely dose-dependent manner. Very important was the finding that CD8+ TCL from the patient demonstrated no proliferative response to any antigenic challenge that was reversed after the addition of exogenous interleukin 2.

Conclusion. Although the identity of the stimulating antigen that led to the expansions observed in this patient is not clarified by the present data, both CD4+ and CD8+ T cells might play a role in the development of ReA following intravesical administration of BCG.

Key words: Reactive arthritis, BCG instillation, T lymphocytes, TCRBV, Peripheral blood, Synovial fluid, Bromodeoxyuridine.

Immunotherapy with Calmette–Guérin bacillus (BCG) is the most effective means currently available for the treatment of superficial bladder cancer [1]. However, this therapy is associated with several side-effects, including joint symptoms [2]. The development of inflammatory arthritis is a rare but well-known complication in patients treated for superficial bladder cancer by intravesical BCG immunotherapy [2]. Although the clinical features of this type of arthritis have been described previously in the medical literature [3], only a few reports have provided data on possible pathogenic mechanisms [4, 5].

A patient who developed inflammatory polyarthritis following intravesical administration of BCG immunotherapy for bladder cancer is reported. A detailed flow cytometry analysis of peripheral blood (PBMC) and synovial fluid mononuclear cells (SFMC) was performed. Furthermore, we studied the response of short-term T-cell lines (TCL) from this patient to different mycobacterial antigens [BCG and tuberculin (purified protein derivative, PPD)] and to human heat shock protein 60 (hsp-60).

Materials and methods

Case report

A 61-yr-old man was admitted to the hospital because of a 1-week history of fever and polyarthritis. He had a previous history of traumatic amputation of his left arm and 2 months prior to admission he was diagnosed as...
having a superficial grade I carcinoma of the bladder. Treatment for his bladder cancer was started with endoscopic resection followed by weekly intravesical BCG immunotherapy for 6 weeks (Immucyst BCG; Inibsa, Barcelona, Spain). One week after the last instillation, he was admitted after complaining of dysuria, malaise, pyrexia of 38.5°C, pain, and swelling in his right hand, knees and right foot. At admission, physical examination revealed arthritis of the right fifth proximal interphalangeal joint, both knees, the right ankle and tarsus, swelling over the right Achilles tendon, and dactylitis in the third finger of his right foot.

Initial laboratory evaluation showed a haemoglobin concentration of 14.1 g/dl, leucocytosis with left shift (11.7 × 10^9/l white blood cells with 79.5% neutrophils), a platelet count of 278 000/mm^2, an erythrocyte sedimentation rate (ESR) of 76 mm/1st h, a C-reactive protein (CRP) concentration of 42.4 mg/dl (normal range 0–0.5 mg/dl) and mildly raised levels of γ-glutamyl transferase (125 U/l, normal range 11–50 U/l). Two series of four blood cultures and the urine culture were sterile. Tests for rheumatoid factor and antinuclear antibodies were negative. The HLA phenotype was HLA-DRB1*08+ and HLA-B27+. The chest X-ray was normal. Examination of SF drawn from the left knee showed a turbid effusion with 64 000 cells/mm^3 with no crystals. SF was sent on two occasions for bacteriological analysis, including mycobacteria [Ziehl-Neelsen, specific cultures and polymerase chain reaction (PCR)], and was always sterile.

The patient was treated with indomethacin for 3 months at 150 mg/day followed by 4 additional months at 100 mg/day. At present, and after 19 months without therapy, the patient remains asymptomatic with no joint signs and normal CRP and ESR.

**Isolation of mononuclear cells and HLA typing**

PBMC were purified from EDTA (ethylenediamine tetraacetic acid)-treated venous blood by Ficoll gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden). DNA was extracted from PBMC with DNAzol reagent (Life Technologies, Grand Island, NY, USA) and HLA-DRB1 alleles were characterized by allele-specific PCR amplification with a Dynal All Set SSP (Dynal, Oslo, Norway). The expression of HLA-B27 was studied by flow cytometry (FACScan; Becton Dickinson, San Jose, CA, USA) using a whole-blood lysis technique with commercially available monoclonal antibodies (mAb) (Becton Dickinson).

**FACS analysis**

PB T-cell phenotype and T-cell receptor (TCR) BV variable region gene products were determined by flow cytometry using a whole-blood lysis technique. The following mAbs were used: FITC (fluorescein isothiocyanate)-conjugated mAbs against CD7, CD45RA, CD45RO, HLA-DR (Pharmingen, San Diego, CA, USA), CD25, CD28, CD57, BV2, BV3S1, BV5S1, BV5S2, BV6S7, BV8, BV13S1/13S3, BV12 and BV17 (Endogen, Woburu, MA, USA); phycoerythrin-conjugated anti-CD4 and -CD8 and FITC-conjugated anti-CD3 (Becton Dickinson). Whole-blood samples were stained within 3 h of venipuncture with two-colour direct immunofluorescence reagents, following the manufacturer’s instructions. Erythrocytes were lysed using FACS® Lysing Solution (Becton Dickinson, San José, CA, USA). Twenty thousand cells were analysed on the day of processing, using a Becton Dickinson FACScanlibur and Simulset software. The panel of mAbs against TCRBV used covered 40–60% of the T-cell repertoire, and BV-restricted expansions were defined as occurring when BV specificities were expressed at a frequency above the mean +3 S.D. of 25 young healthy donors.

**T-cell proliferation assays**

Short-term TCL were established from PBMC by incubating 1 × 10^6 cells for 48 h with immobilized anti-CD3 (5 μg/ml), followed by stimulation with recombinant human interleukin 2 (IL-2) at 20 IU/ml every 48 h. After 2 weeks in IL-2-dependent culture, T cells were harvested, washed in IL-2-free medium to deplete exogenous IL-2, and stimulated in 24-well flat-bottomed culture plates at a concentration of 1 × 10^6 cells/well with the following antigens: BCG, tuberculin (PPD; Evans Medical, Madrid, Spain) and human hsp-60 (Sigma-Aldrich, St Louis, MO, USA). Tetanus toxoid (TT; Evans Medical) was employed as a control for T‑dependent antigen stimulation. The proliferative responses were assessed by measuring bromodeoxyuridine (BrdU) incorporation after 7 days of culture, as described elsewhere [6]. After incubation, cells were collected and stained with an anti-BrdU mAb conjugated to FITC together with a PE-coupled anti-CD4 or -CD8 mAb in the presence of DNase. Thereafter, at least 20 000 cells were analysed by flow cytometry. Results were calculated as signal-to-noise (S/N) ratio using the following formula: % BrdU^+ cells after stimulation/ % BrdU^+ cells without stimulation. We also kept the stimulated TCL for an additional week in IL-2-dependent culture to analyse possible changes in the TCRBV repertoire with respect to that of basal TCL before stimulation.

**Results**

**Distribution and phenotype of SF and PB cells**

In the SF, a marked increase in lymphocytes was detected, mostly due to the CD4^+ T cells, with a CD4/CD8 ratio of 2.95. In contrast, there was lymphopenia in the PB of both CD4^+ and CD8^+ T cell subsets at the onset of active arthritis, and the lymphopenia recovered after clinical remission (Table 1). Flow cytometry analysis also revealed an increase in blood cytolytic T cells (CD3^+ /CD16^−/CD56^−) at the time of the arthritis episode, and this had decreased by about 30% when symptoms had disappeared (Table 1). On the other hand, we also observed a significant increase in granulocytes in PB (9301 granulocytes/mm^3) and SF...
(59 456 granulocytes/mm³) at the onset of arthritis. This abnormality disappeared when symptoms remitted (3924 granulocytes/mm³).

We next analysed the phenotype of PB T cells. The expression of the different markers in PB CD4⁺ and CD8⁺ T cells did not show any significant deviation when compared with aged-paired control subjects. Besides, the frequencies of the different subsets of PB T cells remained unchanged after clinical recovery (data not shown).

In contrast to the PB cells, the SF cells showed an activated memory phenotype in both CD4⁺ and CD8⁺ T-cell subsets. As shown in Fig. 1, within the SF

<table>
<thead>
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<th>Cell subset</th>
<th>PBMC time 0</th>
<th>PBMC time 1</th>
<th>SFMC</th>
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<tr>
<td>CD3⁺/CD5⁺</td>
<td>55.8 (653)</td>
<td>74.62 (1628)</td>
<td>95.5 (4340)</td>
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<tr>
<td>CD3⁺/CD4⁺</td>
<td>42.84 (501)</td>
<td>47.07 (1027)</td>
<td>69.2 (3144)</td>
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<td>CD3⁺/CD8⁺</td>
<td>21.09 (247)</td>
<td>28.24 (616)</td>
<td>23.42 (1064)</td>
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<tr>
<td>CD3⁺/CD16⁺/CD56⁺</td>
<td>22.21 (260)</td>
<td>11.58 (253)</td>
<td>1.35 (61)</td>
</tr>
<tr>
<td>CD3⁺/CD16⁺/CD56⁺</td>
<td>4.12 (48)</td>
<td>1.53 (33)</td>
<td>0.7 (32)</td>
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</tbody>
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Data are percentage (cells/mm³).

*Time 0 was at the onset of arthritis and time 1 after 4 months of clinical remission.

**Table 1. Distribution of peripheral blood (PBMC) and synovial fluid mononuclear cells (SFMC) in a patient with ReA secondary to BCG instillation**

**Fig. 1. Phenotype and TCR BV usage of peripheral blood (black bars) and synovial fluid (grey bars) T cells from a patient with ReA secondary to BCG instillation.** The T cell phenotype (left) and TCRBV gene products (right) of CD4⁺ (A) and CD8⁺ (B) T cells were studied by staining with specific mAbs and flow cytometry analysis. Expansions are indicated by asterisks; they were considered as existing when the frequency of expression of a particular BV family was above the mean + 3 S.D. of the frequency in 25 young healthy donors. SFMC, synovial fluid mononuclear cells.
compartment there was a higher level of expression of CD25+, CD28+ and CD45RO+ T cells. There was no expression of CD45RA in CD4+ T cells and only 19% of the CD8+ T cells were CD45RA+. Parallel to the low levels of NK cells in SF, the proportion of SF CD8+ T cells with a cytolytic phenotype (CD57+) was decreased with respect to PB.

**TCR expression in PB and SF cells**

The initial analysis of TCRBV expression in CD4+ T cells did not show important differences between PB and SF (Fig. 1). However, the frequencies of BV3, BV5S1, BV5S2/SS3, BV8 and BV13.1 were higher in SF CD8+ T cells than in PB cells. In contrast, BV17 was more frequently expressed in PB than in SF. Furthermore, we were able to detect expansions of CD8+ T cells in PB (BV17) and in SF (BV5S1 and BV12). Strikingly, the percentage of PB CD8+ T cells with TCRBV17 diminished (from 11.7 to 5.6%) when the symptoms remitted after 4 months of therapy. It was noticeable that, of the five TCRBV families that expressed higher frequencies in SF CD8+ T cells, three (BV3, BV5S1 and BV5S2/SS3) also decreased in PB CD8+ T cells during the follow-up (BV3, 2.1 to 1.7%; BV5S1, 3.1 to 2.05%; BV5S2/SS3, 4.1 to 2.3%).

**Proliferative response of TCL to various mycobacteria-derived antigens**

Short-term TCL from the patient and a sex- and age-matched control subject were tested in parallel and in duplicate for their proliferative response to BCG, PPD, human hsp-60 and TT (Fig. 2). CD4+ TCL from both the patient and the control subject responded to BCG in a dose-dependent manner, although the response was stronger in the healthy control. The response of CD4+ TCL to PPD in both cases was to the lowest of the stimuli used and, as for BCG, was more intense in the TCL to PPD in both cases was to the lowest of the stimuli used and, as for BCG, was more intense in the TB control. However, CD8+ TCL from both the patient and the control subject responded efficiently to every antigen tested (Fig. 2). CD4+ in a dose-dependent manner, although the response was stronger in the healthy control. The response of CD4+ TCL to PPD in both cases was to the lowest of the stimuli used and, as for BCG, was more intense in the PB and SF of a patient with active reactive arthritis (ReA) after BCG immunotherapy for bladder cancer. Furthermore, the response of patient’s T cells to several mycobacteria-derived antigens in vitro reveals the potential role of both CD4+ and CD8+ T-cell subsets in the development of this ReA.

Although CD4+ T lymphocytes were augmented in the SF of the patient, they did not show any significant expansion. However, the CD8+ subset displayed several expansions in both the PB and SF at onset of arthritis that decreased after remission, suggesting a possible role of these lymphocytes in the development of ReA after BCG instillation, as described for other ReA [7, 8]. However, it is well known that expansions in CD8+ T cells are more common than in CD4+ T cells in both diseased and healthy aged subjects [9–12]. The phenomenon of T-cell expansion may reflect the proliferation of antigen-specific T cells, a non-specific expansion of memory T cells in response to the production of inflammatory cytokines, or a defect in T-cell homeostasis that may predispose to the disease. We consider that our results support the first hypothesis for several reasons. First, the SF CD8+ T cells showed a phenotype of activated and memory cells, as described in ReA caused by other infectious agents [7, 13]. Secondly, the BV-restricted expansions, together with the recent activation phenotype, indicate a specific stimulation of CD8+ T cells by the BCG [14]. Thirdly, in vitro stimulation with mycobacteria-derived antigens induced a higher expression of some BV families expanded in PB and SF CD8+ T cells. Although we cannot exclude non-specific T-cell proliferation in response to pro-inflammatory cytokines, it is also important to remark that the major TCRBV expansions observed decreased after remission of the symptoms, making the third hypothesis less probable. This can be explained by the acute and limited course of the disease, in contrast to the persistence of TCRBV expansions described in other chronic forms of ReA [15].
On the other hand, the strong in vitro response of CD4+ TCL from the patient to mycobacterial antigens indicates their antigen-specificity and reactivity. A possible explanation is that these lymphocytes would have a helper effect by secreting cytokines that stimulate the antigen-specific CD8+ T-cell response [16]. In contrast, the in vitro stimulation of CD8+ TCL showed a state of unresponsiveness to every antigen tested. This lack of responsiveness, which was reversed after the addition of exogenous IL-2, is compatible with the state of anergy. Some authors suggest that such anergy may be the result of presentation of self-antigens by non-professional antigen-presenting cells or the influence of locally produced cytokines [17]. Indeed, some reports have shown an ineffective Th1 response, which would contribute to bacterial persistence [18]. At present, it is argued that IL-10-secreting T-cell clones act as regulator T cells that could down-regulate T CD8+ activity [19].

Mycobacteria are intracellular pathogens that are eliminated by the cytolytic response of CD8+ cells. In this response, the antigen must be presented by HLA-I molecules [20]. In the case of mycobacteria-associated ReA after intravesical therapy, there is a clear association

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**Fig. 2.** Proliferation of CD4+ (left) and CD8+ (right) TCL obtained from peripheral blood of the patient with ReA (grey bars) and one aged-matched control subject (white bars). As described in Materials and methods, TCL were stimulated for 72 days with BCG, PPD and hsp-60 at 5, 2.5 and 1 μg/ml. TT was employed as control for stimulation. Proliferating cells were detected by staining with an anti-BrdU mAb after incorporation of BrdU. Results are expressed as signal-to-noise (S/N) ratio, calculated as the percentage of BrdU+ cells after stimulation divided by the percentage of BrdU+ cells without stimulation.
with HLA-B27 [21]. Our patient carried the HLA-B27 antigen, which could increase the sensitivity of lymphocytes to certain bacterial antigens [22]. Experimental work has shown cross-reactivity between mycobacterial antigens and cartilage proteins. Furthermore, hsp are major antigenic determinants and mycobacterial hsp have sequence homology with human hsp [23]. All these data together suggest the existence of a cytotoxic response triggered by BCG instillation that could be specific for HLA-B27 molecules carrying endogenous peptides, such as human hsp-60, by a molecular mimicry mechanism [15]. In fact, Zugel et al. [24] considered cross-reactivity between hsp-60 from Mycobacterium tuberculosis and human hsp-60 to be necessary in order to induce autoimmune disease. In our patient, an immune response initiated by BCG instillation may have been enhanced by cross-reactivity with the patient’s self-hsp, which was presented by the HLA-B27 molecule.

In summary, BCG instillation might be responsible for the activation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Thereafter, these T cells could cross-react with self-antigens, such as hsp-60. However, the present data do not clarify the nature of the antigen responsible for the activation and BV-restricted expansions described.

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References


