T Cells From Patients With Hodgkin’s Disease Have a Defective T-Cell Receptor ζ Chain Expression That Is Reversible by T-Cell Stimulation With CD3 and CD28

By Christoph Renner, Sascha Ohnesorge, Gerhard Held, Stefan Bauer, Wolfram Jung, Jan-Peter Pfitzenmeier, and Michael Pfreundschuh

To investigate the mechanisms underlying the deficiency of T lymphocytes from patients with Hodgkin’s disease, we investigated the expression of the T-cell receptor (TCR)ζ chain in patients with Hodgkin’s disease. By flow cytometry using an anti-ζ chain monoclonal antibody, peripheral blood T lymphocytes from patients with untreated Hodgkin’s disease were shown to express decreased levels of the TCRζ chain. After stimulation by combined CD3 and CD28 cross-linking, T cells from Hodgkin’s disease patients upregulated ζ chain protein expression to normal values within 48 hours and achieved a cytolytic potential and levels of interleukin (IL)-2 secretion that were not different from T cells obtained from healthy controls. These results show that downregulation of the TCRζ chain in Hodgkin’s T lymphocytes is a reversible event. Co-stimulation of CD3 and CD28 is a novel approach for overcoming the T-cell deficiency in Hodgkin’s disease and might be exploited clinically. As upregulation of the ζ chain can also be achieved using bispecific monoclonal antibodies (Bi-MoAbs) with specificity for tumor antigens and CD3 and CD28, respectively, an immunotherapy with CD3/CD30 and CD28/CD30 Bi-MoAbs may overcome and should, therefore, not be jeopardized by the inherent T-cell deficiency in patients with Hodgkin’s disease.

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The increased frequency of viral and fungal infections and the decreased delayed type hypersensitivity reaction in patients with Hodgkin’s disease have been known for a long time. Patients with Hodgkin’s disease were shown to be anergic against standard recall antigens and to exhibit a diminished rejection reaction of allogeneic skin transplants. A vast body of literature has accumulated on different aspects of the depressed T lymphocyte response in vitro to phytohemagglutinin (PHA), concavalin A (Con A), and pokeweed mitogen. These functional abnormalities showed some correlation with the severity of the disease and were reported to have prognostic relevance. As Hodgkin’s disease patients did not differ significantly from healthy controls with regard to their lymphocyte counts, the T-cell deficiency in Hodgkin’s disease was assumed to be caused by qualitative defects, such as a decreased proliferative response on stimulation with standard mitogens and the secretion of significantly lower amounts of interleukin (IL)-2. The latter could not be assigned to a compromised expression of the IL-2 receptor (IL-2R) in the T cells from Hodgkin’s patients. The IL-2R was found to be in the normal range. The hypothesis that Hodgkin’s patients have an intrinsic defect for enzymes with relevance for T-cell function was supported by observations of a decreased activity of the enzymes adenosine deaminase and 5’-nucleotidase in Hodgkin’s T cells, which are essential for adequate T-cell proliferation. As the same enzyme defect can be detected in other malignant diseases that are associated with clinically relevant immunodeficiencies and because Epstein-Barr virus (EBV)-positive tumors are the underlying disease in most of these cases, it was speculated that the EBV infection was the common causative link for the observed immunodeficiency.

In previous studies, we had developed a model for the treatment of Hodgkin’s disease using the combination of two T-cell activating bispecific monoclonal antibodies (Bi-MoAbs), which recognize one arm the Hodgkin’s associated CD30 antigen and with the other, the CD3 or CD28 antigen on T cells, respectively. We showed that even severe combined immunodeficient (SCID) mice bearing disseminated Hodgkin’s lymphoma can be cured using the combination of CD3/CD30 and CD28/CD30 Bi-MoAbs and normal human T cells. When testing different sources of T cells in this model, the T cells from patients with Hodgkin’s disease turned out to be as efficient as those from normal controls, even though they showed the previously described deficits in proliferative responses to mitogens, suggesting that the cross-linking of the T cells by the CD3 and CD28 Bi-MoAbs in the presence of the tumor cells overcomes or compensates for the mechanisms underlying the T-cell deficiency in Hodgkin’s disease.

A prime candidate to be involved in this deficiency was, therefore, T-cell receptor (TCR)ζ chain. The necessity for the ζ chain in TCR-mediated signaling had been shown by studies of a ζ chain negative mutant derivative of an antigen-specific T-cell hybridoma. The mutant cell line was incapable of responding to antigen and was only poorly responsive to anti-CD3 antibodies. Experiments where ζ chain deletion variants caused defects in signal transduction in transfected cell lines clearly pointed to an important, if not primary, role for the ζ chain in signal transduction. Moreover, two groups have reported independently that T cells from cancer patients with clinically obvious signs of reduced immune activity have significantly decreased expression levels of the TCR signal transduction molecule CD3ζ.

It was, therefore, the aim of this study to investigate whether there is indeed a reduced ζ chain expression in patients with untreated Hodgkin’s disease and whether combined CD3 and CD28 stimulation can overcome this defect to an extent where normal levels of T-cell effector function are achieved.
T-CELL RECEPTOR ζ-CHAIN IN HODGKIN'S DISEASE

Table 1. Characteristics of Hodgkin’s Patients Studied

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<th>Characteristics of Hodgkin’s Patients Studied</th>
<th>Total</th>
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<th>IL-2 Secretion</th>
<th>Cytotoxicity</th>
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MATERIALS AND METHODS

Antibodies and cell lines. The generation, purification, and characterization of the Bi-MoAbs (anti-CD3/CD30 and anti-CD28/CD30) has been described previously. Established human Hodgkin’s-derived cell lines used in this study have been described elsewhere. The IL-2-dependent CTLL2 mouse hybridoma was obtained from American Type Culture Collection (Rockville, MD). The monoclonal antibodies (MoAbs) used in this study for fluorescence-activated cell sorter (FACS) analysis and immunomagnetic selection were: anti-CD2, anti-CD3, anti-CD7, anti-CD19, anti-CD45 (DAKO, Hamburg, Germany), anti-CD3-FTTC/PE, anti-CD14-FTTC, anti-HLA-DR-PE, anti-CD45RA-FTTC (Becton Dickinson, Heidelberg, Germany), and anti-TCR-zeta TIA-2 (Coulter, Hialeah, FL).

Patient characteristics. A total of 105 blood samples from patients with untreated Hodgkin’s disease were collected. All blood samples were kept frozen until the experiment was performed and used only when the viability of the T cells after thawing was >90%. The patients’ characteristics are listed in Table 1.

T-cell preparations. A total of 40 to 50 mL EDTA blood were collected from Hodgkin’s disease patients before treatment and peripheral blood mononuclear cells (PBMC) were isolated as described. After lysis-methylene-ester (LME) treatment, T cells were negatively enriched by immunomagnetic depletion of CD14, CD16, CD19, and HLA-DR expressing cells using a VarioMACS system (Miltenyi, Bergisch Gladbach, Germany). The remaining lymphocytes were >95% CD3+. Contaminating cell fractions were always <0.5%; no proliferation after phorbol myristate acetate (PMA) (10 ng/mL) or PHA (1 μg/mL) stimulation for 2 to 5 days in culture was observed.

FACS analysis. For intracellular staining of the TCR ζ chain, cells were permeabilized by saponin (Sigma, Munich, Germany) and FACS analysis was performed according to the method described by Assenmacher et al. A fluorescein isothiocyanate (FITC) conjugated goat antimouse F(ab’2) Ig monomer antibody was used as second step reagent (Dianova, Hamburg, Germany). TCR ζ chain expression was measured as described by others using the CD3ζ/CD3ε ratio for the exact evaluation of the balance between these two TCR associated chains.

Activation of T lymphocytes. For T-cell activation, CD3 (1 μg/mL UCHT1; Dako, Hamburg, Germany) and CD28 MoAb (2 μg/mL 15E8) were coated to 24-well plates. The plates were washed twice with phosphate-buffered saline (PBS) followed by PBS with 1% gelatine to block unspecific binding of antibodies (37°C, 1 hour) and purified T cells (2 x 10⁶ cells/mL) added for the indicated time. In the case of cytotoxicity experiments, 24-well plates (Nunc, Nürtingen, Germany) were coated with CD30-FP (1 μg/mL) in PBS at 4°C overnight. T cells were activated in the presence of both CD3/CD30 and CD28/CD30 Bi-MoAbs (200 ng/mL) as described and used for further experiments.

Assay for cytolytic activity. Bi-MoAb-mediated cytotoxicity was measured using the technique of time resolved fluorometry with the described modifications. The Hodgkin’s-derived cell lines L540CY and HDLM2 were used as targets for Bi-MoAb-activated T cells. Fresh Bi-MoAb (1 μg/mL) was added at the onset of the experiment. The E/T ratios were used in the indicated range. The cytotoxicity assay was stopped after 4 hours and analyzed as described.

IL-2 release assay. To determine the IL-2 release of Bi-MoAb activated T cells obtained from healthy donors and Hodgkin’s disease patients, supernatant from 1 x 10⁶ unstimulated lymphocytes or stimulated in the presence of both Bi-MoAbs (2 x 10⁶ ng/mL) for 3 days was collected and added to 1 x 10⁶ CTLL2 cells, which were grown in IL-2-free medium for 1 day. Twenty-four hours later, cell cycle activity was measured using a EZ4U kit (Biozol, Munich, Germany). Units of IL-2 were determined by comparing two standards obtained from recombinant IL-2 at different doses.

Determination of intracellular free calcium concentration. Analysis of the intracellular free calcium concentration was performed as described using Fura-2 acetoxymethylester (Sigma, Munich, Germany). After staining, resting or Bi-MoAb-activated lymphocytes were resuspended in 2 mL of a buffer consisting of 150 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L MgCl₂, 10 mmol/L glucose, 10 mmol/L MOPS (3-[N-Morpholino] propane-sulfonic acid), 0.3% bovine serum albumin (BSA), and 1 mmol/L CaCl₂. Fluorescence measurements were made using a spectrofluorometer (Perkin Elmer model LS-50; Perkin Elmer, Heidelberg, Germany) at an excitation wavelength of 335 nm and an emission wavelength at 510 nm under constant stirring. Cells were equilibrated at 37°C until a baseline was reached when anti-CD3 MoAb (10 μg/mL) was added. Antibody cross-linking was induced by the addition of goat antimouse antibody (30 μg/mL; Dako, Hamburg, Germany). At the termination of the response, 20 μL 10% Triton X-100 was added to determine Rmax followed by 100 μL 0.5 mmol/L EGTA to determine Rmin. [Ca²⁺], was calculated using the formula:
**RESULTS**

**TCRζ chain expression in the PB of patients with Hodgkin’s disease.** When T cells from healthy donors and Hodgkin’s disease patients were compared, the patients’ T cells exhibited a pronounced and significantly decreased expression level of the TCRζ chain protein. To standardize the intensities of ζ chain expression in T cells from different donors and patients, the expression of the CD3ε and the CD3ζ chain was measured simultaneously, and the ratio of these two parameters was determined. As shown in Fig 1, normal donors had a zeta/epsilon ratio of 0.96 (Fig 1A). In contrast, Hodgkin’s disease patients had a significantly decreased ratio of 0.37 (Fig 1B; P value <.01 in the student’s t-test). The intensity of the CD3 staining per cell and the expression level of the CD3ε chain was not different when normal donors were compared with patients (Fig 2).

**Upregulation of the TCRζ chain in patients’ T cells by combined CD3 and CD28 cross-linking.** Simultaneous CD3 and CD28 antigen cross-linking mediated by respective parental CD3 and CD28 MoAbs or respective Bi-MoAbs (Fig 3) in the presence of CD30-FP resulted in the upregulation of the ζ chain to normal values in the patients’ T cells within 48 hours. Twenty-four hours after combined T-cell activation, the protein expression of the TCR ζ chain increased and reached normal values after 2 days (Fig 4). The difference of TCR ζ chain expression in resting and activated T lymphocytes by this procedure was highly significant (P < .01). Cross-linking by one Bi-MoAb or parental antibody alone had no effect on the TCRζ chain expression. Expression levels of the ζ chain in healthy donors were not significantly increased by this procedure (data not shown).

**Ca²⁺ mobilization in Hodgkin T cells.** Resting T lymphocytes from untreated Hodgkin’s disease patients had an impaired Ca²⁺ mobilization after CD3 antigen crosslinking (Fig 5). In contrast to T cells from healthy controls that reacted with a prompt and significant increase of [Ca²⁺]i after antigen cross-linking, the patients’ T cells showed only a slight increase of [Ca²⁺]i after CD3 stimulation. However, after achieving normal TCR ζ chain expression levels by

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\[ [\text{Ca}^{2+}]_i = K_d \cdot \frac{(F - F_{\text{Min}})}{(F_{\text{Max}} - F)} \]

with K_d = 224 nmol/L and F = measured fluorescence.
combined CD3 and CD28 antigen stimulation for 3 days, the \([Ca^{2+}]\), mobilization in the patients' T cells was no longer significantly different from the one obtained in healthy donors. Again, the difference in \([Ca^{2+}]\), mobilization of resting and activated T lymphocytes from Hodgkin's disease patients was highly significant \((P < .005)\).

**IL-2 secretion by T cells from Hodgkin's disease patients.** Restoration of \(\zeta\) chain expression and normalization of the \([Ca^{2+}]\), mobilization in patients' T cells associated with the secretion of normal amounts of IL-2 by activated lymphocytes (Fig 6). After adequate stimulation with both CD3 and CD28 Bi-MoAbs or the parental bivalent CD3 and CD28 MoAbs for 3 days, the patients' T cells secreted levels of IL-2 that were comparable to the ones obtained from healthy controls, as determined by the proliferation of the IL-2–dependent CTLL2 tumor cell line cultured in the supernatant from activated lymphocytes. One MoAb alone had no significant impact on level of IL-2 secretion. T cells stimulated for 1 day provided still depressed levels of TCR\(\zeta\) chain expression and secreted significantly lower amounts of IL-2 when compared with T cells stimulated for at least 48 hours.

**T-cell–mediated cytotoxicity.** In a next step, T cells from Hodgkin's disease patients were used for cytotoxicity assays after stimulation with a combination of both CD3/CD30 and CD28/CD30 Bi-MoAbs. There was no significant difference in Bi-MoAb–mediated cytotoxic activity against the CD30+ Hodgkin's-derived tumor cell lines between the patients' T cells and the T cells from healthy controls (Fig 7). The Bi-MoAb–mediated tumor cell lysis was strictly antigen-dependent, as only CD30+ tumor cell lines were killed. Even at low lymphocyte concentrations (E:T ratio), T lymphocytes

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**Fig 4.** Kinetics of TCR\(\zeta\) chain restoration in T cells from patients with Hodgkin's disease. The protein expression of the \(\zeta\) chain was upregulated to normal levels only in the group activated with a combination of the CD3 and CD28 Bi-MoAb (●). No control antibody [CD 3 (●) or CD28 (●)] alone had a similar effect. Five patients were used per group. SD is indicated by the bars.

**Fig 5.** \([Ca^{2+}]\), mobilization in resting (groups 1 and 3) and Bi-MoAb–activated (groups 2 and 4) T cells from 25 patients with Hodgkin's disease (groups 1 and 2) and 25 healthy controls (groups 3 and 4). [Ca\(^{2+}\)], mobilization was induced by cross-linking of a CD3 antigen specific MoAb followed by the addition of polyclonal goat antimouse serum. Results are shown as the median of 25 patients and 25 healthy controls; bars indicate SD.

**Fig 6.** IL-2 secretion of T cells (20 Hodgkin's patients, 20 healthy controls) before and after stimulation by combined CD3 and CD28 cross-linking. The IL-2 secretion was determined in a proliferation assay with the IL-2–dependent cell line CTL2. 1, medium alone; 2, unstimulated; 3, stimulated T cells from Hodgkin's patients; 4, unstimulated; 5, stimulated T cells from healthy controls. Results are shown as the median ± SD of 20 individuals per group. The difference of IL-2 secretion in resting and preactivated T cells from Hodgkin patients was highly significant \((P < .01)\).

**Fig 7.** Bi-MoAb–mediated cytotoxicity of T cells. T cells from healthy donors (●) and patients with untreated Hodgkin's disease (●) were used immediately (open symbols) or after stimulation (closed symbols) with a combination of CD3/CD30 and CD28/CD30 Bi-MoAbs for 3 days as cytotoxic effector cells against the Hodgkin's–derived L540CY cell line. Results are shown as the median ± SD of 20 individuals in each group.
from Hodgkin’s disease patients were as cytotoxic as the controls. T-cell activation with consecutive tumor cell lysis was not achieved if one MoAb alone was used for stimulation.

DISCUSSION

Our study on the T-cell deficiency in untreated patients with Hodgkin’s disease yielded two important results: it is the first description of a defect in \( \zeta \) chain expression in peripheral T cells from patients with Hodgkin’s disease, and it is the first to describe a strategy to overcome this defect. The original report on a decreased TCR \( \zeta \) chain expression in malignant disease came from Mizoguchi et al.\(^{25}\) who observed low amounts of CD3\( \alpha \) and CD3\( \zeta \) chains and decreased amounts of p56\( \text{Stat} \) and p59\( \text{Stat} \) TCR/CD3 associated kinases in the TCR/CD3 complexes of tumor bearing mice. The decreased expression of these molecules, which are all involved in signal transduction, resulted in an abnormal activation through the TCR/CD3 complex and in depressed T-cell responses. Meanwhile, two groups have confirmed this observation for human tumors.\(^{19,20}\) They described low CD3\( \zeta \) levels in tumor infiltrating lymphocytes (TIL) and, to a lesser extent, PB T lymphocytes from patients with colorectal or renal cell carcinoma. In our study of untreated patients with Hodgkin’s disease, we standardized the intensities of the \( \xi \) chain expression in T cells from different donors and patients by determining the ratio of the CD3\( \varepsilon \) and the CD3\( \zeta \) chain expression. The fact that the amount of CD3\( \varepsilon \) molecules per T cell was not significantly different in patients and controls shows that only distinct molecules are affected in Hodgkin’s-associated T-cell deficiency and not the TCR/CD3 complex as a whole. The downregulation of the TCR\( \xi \) chain in T cells from tumor-bearing patients might be a widespread phenomenon in both animal and human cancers and might be a means of the tumor to escape an immune response.\(^{26}\)

The level of TCR\( \xi \) chain expression might correlate with disease activity, as has been suggested by a recently published case report from a patient with B-cell lymphoma. While the \( \zeta \) chain was expressed at very low levels in the PB T cells of that patient at the time of diagnosis, the expression of this molecule raised to almost normal levels after successful treatment.\(^{24}\) The study of additional patients and a longer follow-up of the patients included in our study will answer the question whether the restoration of the \( \zeta \) chain to normal values does indeed follow and depend on the successful treatment of the underlying malignant disease, and if patients with a higher expression level of the TCR \( \zeta \) chain may have a more favorable outcome of the disease. More patients need to be studied to obtain valid data on the question if there is a significant correlation between the stage of disease at the time of diagnosis and the expression level of the TCR\( \xi \) chain.

The decreased TCR\( \xi \) chain expression in peripheral blood T cells from patients with untreated Hodgkin’s disease is associated with an altered signal transduction in these cells as shown by the impaired mobilization of [Ca\( ^{2+} \)]. Whether the defective TCR\( \xi \) chain expression is the only reason for the inadequate T-cell responses and partial T-cell anergy in Hodgkin’s disease patients is currently under investigation, as are the mechanisms that are responsible for the downregulation of the \( \zeta \) chain. Preliminary results suggest that cytokines such as transforming growth factor (TGF\( \beta \)) may play a major role in this process.

Our finding that the defective TCR \( \zeta \) chain expression can be restored by a T-cell stimulation via combined CD3 and CD28 antigen cross-linking offers a strategy for the circumvention of what seems to be quite a common tumor-associated immune deficiency in patients with malignant disease. T cells from Hodgkin patients activated by this procedure upregulate the TCR \( \zeta \) chain within 48 hours to normal levels. The upregulation of this TCR-associated chain correlates with the effector function of these T cells as normalization of IL-2 secretion and cell-mediated cytotoxicity parallels the restoration of \( \zeta \) chain expression. Other strategies for overcoming the \( \zeta \) chain defect and restoring an efficient cellular response in the respective patients with tumor, eg, by treatment with IL-2, have met with limited success.\(^{27}\) The fact that the upregulation of the TCR\( \xi \) chain in T cells from these patients by combined CD3 and CD28 stimulation restores defective signal transduction (as shown by normalization of the Ca mobilization) and results in normal effector function (as shown by normal T-cell-mediated tumor cell lysis and cytokine secretion) implies that the TCR \( \zeta \) chain is critically involved in the T-cell deficiency in patients with Hodgkin’s disease. The fact that only the activation of T cells by simultaneous CD3 and CD28 stimulation is able to upregulate the defective expression of the TCR\( \xi \) chain supports the concept that two signals are necessary for complete T-cell activation, which includes a normal proliferative response and cytotoxic capacity.\(^{28}\) This is of importance for the design of immunotherapeutic concepts for the treatment of cancer, as many tumors are deficient in costimulatory molecules and might not be capable to prime naive T cells, even if they presented tumor-specific antigenic peptides in the context of the major histocompatibility complex (MHC) molecules on their surface that could be recognized by the patient’s T cells. According to the results reported here, the one signal provided by the antigenic tumor-specific peptide alone would not be able to overcome the tumor-associated T-cell deficiency mediated via decreased \( \zeta \) chain expression and would result in further T-cell anergy. Rather, it seems critical to deliver a second signal, which in the case of CD28 stimulation, should not only overcome T-cell anergy, but would also be instrumental in restoring the tumor-associated decrease in \( \zeta \) chain expression.\(^{29}\) Therapeutic strategies aimed at this include the transfection of tumor cells with costimulatory molecules such as B7-1 or B7-2 or the use of a combination of two Bi-MoAbs, which bind to both a tumor-associated antigen and the CD3 or CD28 antigen on T cells, respectively.\(^{30,31}\) While the first strategy, which involves all the logistic problems of gene therapy, is cumbersome and expensive and awaits the resolution of basic technical problems, the latter approach promises to be easily transferable to the clinical situation. Indeed, after having established a model for the treatment of human tumors in SCID mice using a combination of two Bi-MoAbs that stimulate T cells via CD3 and CD28,\(^{32}\) and having demonstrated that this model is effective for the treatment of SCID
mice with disseminated and advanced human tumors, the demonstration in this report that the patients’ lymphocytes, despite a preexisting, yet restorable deficiency of the TCRζ chain function normally in terms of signal transduction measured by calcium mobilization, proliferation, cytokine secretion, and cellular cytotoxicity confirms the need for both Bi-MoAbs in such an approach and encourages a clinical trial of this strategy in patients whose tumors are resistant to conventional treatment.

ACKNOWLEDGMENT

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