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Chemoimmunotherapy for melanoma with dacarbazine and 2,4-dinitrochlorobenzene elicits a specific T cell-dependent immune response

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Abstract An empirically established chemoimmunotherapy for metastatic melanoma combines the systemic administration of the chemotherapeutic agent dacarbazine (DTIC) with the epifocal application of the contact sensitizer 2,4-dinitrochlorobenzene (DNCB) on cutaneous metastases. Although this therapy yields high response rates resulting in prolonged survival, the mechanisms involved remain unknown. Here, we investigated whether treatment of tumor-bearing mice with DTIC and DNCB resulted in a specific immune response against the tumor. Subcutaneous (s.c.) tumors and lung metastases were induced in C57BL/6 mice by injecting syngeneic B16-melanoma cells s.c. or into the lateral tail vein, respectively. Mice were treated with intraperitoneal injections of DTIC followed by epifocal application of DNCB. This therapeutic approach significantly reduced the growth of s.c. tumors as well as lung metastases. Our data showed that the effector mechanisms involved are dependent on T cells. No therapeutic effect was observed in immunodeficient RAG-$1^{-/-}$ mice, or when the contact sensitizer DNCB was replaced by skin irritants (croton oil or tributyltin). Splenic lymphocytes obtained from treated mice displayed a three-fold higher specific cytolytic activity against B16 cells than in tumor-bearing controls. Both $CD8^+$ and $CD4^+$ T cells were able to lyse B16 cells. No changes were observed in natural killer (NK) cell activity. Likewise, tumor-infiltrating lymphocytes (TIL) of treated mice showed higher cytolytic activity than that of controls. Analysis of cytokine expression in s.c. tumors revealed increased mRNA levels of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) in treated tumors. Together, these findings demonstrate the ability of DTIC/DNCB treatment to induce an effective T cell-dependent host immune response against a syngeneic tumor.

Keywords Cytotoxicity · Tumor immunity · T lymphocyte

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Introduction

Since malignant melanoma is known to be an immunogenic type of tumor, infiltration of the latter with immunocompetent cells such as T lymphocytes and a number of clinical phenomena such as regression of the primary tumor illustrate the ongoing immunological reaction against this tumor [1, 54]. Characterization of tumor-infiltrating lymphocytes (TIL) indicates that a variety of antigens expressed by melanoma tumor cells, so-called melanoma-associated antigens, can be recognized by the immune system. However, most progressively growing tumors including melanoma do not provoke antitumor-immune responses that are capable controlling the growth of malignant cells [41]. Accumulating evidence exists that tumor cells may downregulate the expression of signals that are necessary for the activation of host T cells. The mechanisms include defective expression of major histocompatibility complex (MHC) molecules and/or costimulatory molecules as well as the release of immunosuppressive cytokines [1, 2, 8, 12, 15, 51, 60]. Thus, the goal of therapeutic approaches is either to enhance an already existing immune response or to induce a specific antitumor response [25]. Strategies to augment the host immune response to tumor have included immunomodulatory therapies, e.g. defined cytokines such as interferon-alpha and interleukin-2 (IL-2), vaccination therapy with dendritic cells or with genetically modified tumor cells as

well as chemoimmunotherapies [18, 25, 26, 37, 44, 45, 48, 50, 56].

Recently, two groups have reported a therapeutic approach which combines the epifocal application of 2,4-dinitrochlorobenzene (DNCB) on cutaneous metastases with the systemic adminisration of dacarbazine (DTIC) [50, 59]. This therapy yields high reponse rates resulting in prolonged survival of the patients. DTIC, a DNA alkylating agent, is the most frequently used single agent for therapy of metastatic melanoma, inducing objective remissions in up to 20% of patients, but the impact on survival is low [10]. DNCB, a potent contact sensitizer, has been used for the treatment of primary and metastatic melanoma since 1973 as an active non-specific immunotherapeutic agent. This approach was abandoned due to toxicity and limited activity [28]. Therefore, the effectiveness of this combined therapy seems to be to due a synergistic effect of these compounds. However, despite the fact that this therapy is in clinical use, its mode of action is still unclear. In order to overcome this limitation we previously established a murine model system. Using the B16-melanoma model we demonstrated that chemoimmunotherapy with DTIC and DNCB results in a decreased growth of subcutaneous (s.c.) tumors [61]. Treatment of animals with DNCB alone did not show any effect on tumor growth when compared with untreated control animals, whereas injection of DTIC alone resulted in slightly though not significantly delayed tumor growth. Furthermore, treatment with DTIC and DNCB also influenced the course of experimentally induced pulmonary metastases. The number of pulmonary foci on the organ surface was reduced significantly by the treatment.

The present study was designed to examine immunologic effector mechanism(s) of chemoimmunotherapy with DTIC and DNCB in a murine tumor model. We have demonstrated that the observed therapeutic effect is mediated via a specific immune response, in which both $CD4^+$ and $CD8^+$ T cells are involved.

Materials and methods

Animals

Female C57BL/6 mice, aged 4–6 weeks old, were purchased from Harlan Winkelmann (Borchen, Germany). C57BL/6 RAG-$1^{-/-}$ mice were obtained from H. Mossmann (Max-Planck-Institut für Immunologie, Freiburg, Germany). The animals were housed under specific pathogen-free conditions and were used for the experiments at the age of 6–12 weeks. All experiments were performed according to the National Institute of Health guidelines for care and use of laboratory animals.

Subcutaneous tumors

The murine melanoma cell line B16FB17, a slowly growing not spontaneously metastatic B16 subline, was used in all experiments. Tumor cells were maintained as monolayers in vitro in complete RPMI 1640 medium, containing 10% fetal calf serum, 2 mM glutamine, streptomycin (100 μg/mg) and penicillin (100 IU/ml) and were passaged as necessary. Tumors were induced by subcutaneous (s.c.) injection of 2×10^6 (25 μl) melanoma cells in the right flank of the mice. Treatment was started 7 days thereafter, when tumors reached an average volume of 25 μl. One treatment cycle consisted of an intraperitoneal (i.p.) injection of DTIC (50 mg/kg) followed after 24 h by an epifocal application of DNCB (25 μl acetone and olive oil, 4:1) on the s.c. tumors. For the first cycle, s.c. tumors were treated with 2% DNCB (sensitization phase of the contact hypersensitivity reaction), whereas for the following cycles 1% DNCB was used (elicitation phase of the contact hypersensitivity reaction). Cycles were repeated 4 times every 4th day. The tumor volume was calculated as half the product of the length times width square.

Experimental lung metastases

Seven days after inoculation of s.c. tumors, a single-cell suspension of 2×10^6 tumor cells in 200 μl Hank's balanced salt solution (HBSS) was injected into the lateral tail vein. Treatment of animals with DTIC and DNCB was started 48 h later (day 9). Treatment cycles consisted of i.p. injection of DTIC (25 mg/kg) followed by an epifocal application of DNCB after 24 h. In total, mice received 7 treatment cycles, one every 4th day. Animals were killed 30 days after the induction of pulmonary metastases. Lungs were removed, fixed in Bouin's solution and examined under a low-magnification microscope for the presense of tumor foci on their surface.

Isolation of TIL

Subcutaneous tumors were harvested aseptically and cut into small pieces followed by treatment with collagenase type III (40 mg/ml; Worthington Biochemicals, Freehold, N.J.) and Dnase I (20 μg/ml; Boehringer Mannheim, Germany) for 2 h at room temperature. The resulting suspension was filtered through a stainless steel mesh. After three washings with phosphate-buffered saline (PBS) suspension applied on 5 ml Lympholyte M was subjected to centrifugation at 1,300 *g* for 25 min. The cells at the interphase were collected, washed three times with PBS and were positively purified using a combination of anti-CD4, anti-CD8, and anti-DX5 MicroBeads and MACS columns (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Purified cells were used as tumor infiltrating lymphocytes (TIL).

Cytotoxicity assay

Mice were killed 7 days after the last treatment. A standard 4-h ^{51}Cr release assay was performed after in vitro stimulation of splenocytes. Single cell suspension of splenocytes (1×10^7/well) were stimulated in vitro with irradiated (15,000 rad) B16-melanoma cells (2×10^5/well) in 24-well culture plates in complete media containing 50 IU/ml recombinant human interleukin-2 (IL-2). After 3 days of mixed tumor–splenocyte culture at 37°C, splenocytes were harvested and subjected to centrifugation over Lympholyte M. In some experiments different T-cell subpopulations were positively purified from splenocytes using anti-CD4, anti-CD8, or anti-DX5 microbeads and MACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany). In each experiment aliquots of isolated cells were analyzed for the expression of specific cell surface markers by flow cytometry. Isolated populations were routinely >95% pure. Effector cells were mixed with ^{51}Cr-labeled target cells at various effector:target (E:T) ratios in a 96-well V-bottomed plate. Target cells (B16, EL-4, and YAC-1) were labeled by incubating them with 100 μCi of ^{51}Cr (ICN Biomedicals, Eschwege, Germany) at 37°C for 90 min. After mixing effectors and targets (in triplicate wells), plates were placed at 37°C for 4 h. Supernatant from each well was harvested and the amount of ^{51}Cr released was determined in a gamma counter. Spontaneous release was obtained from supernatants of target cells cultured without effector cells. Maximum release was obtained from supernatants of target cells after lysis with Triton X-100. Specific release was determined using the following equation: experimental cpm–spontaneous cpm)/(maximum cpm–spontaneous cpm)×100.

TIL (1.2×10^5/well) were stimulated in vitro with irradiated (15,000 rad) B16-melanoma cells (4×10^4/well) in 96-well culture plates in complete media containing 50 IU/ml recombinant human IL-2. After 3 days of mixed tumor–lymphocyte culture at 37°C, lymphocytes were harvested and depleted of dead cells by Ficoll gradient centrifugation. The cytolytic activity of in vitro restimulated TIL against syngeneic B16-melanoma cells was analyzed using a microcytotoxicity assay modified according to the method of Bröcker et al. [7]. One thousand target cells (B16-melanoma cells) per well plated on Terasaki plates in 10 µl RPMI medium and incubated overnight. After removal of the medium, effector cells (TIL) were cocultured with targets at indicated E:T ratios for 18 h. To remove effector cells and lysed cells, plates were rinsed twice with prewarmed PBS. Subsequently, plates were fixed and stained according to the method of Pappenheim. Only cells with obvious nuclei were counted. Lysis was quantified by determination of B16 melanoma cell reduction (control–probe), and expressed as percentage of control.

RNA isolation and RNase protection assay

Total RNA from s.c. B16 tumors was isolated by the method of Chomczynski and Sacchi [14]. RNase protection assays were performed using RPA kits and the multiprobe template sets mCK-1, mCK-2, and mCK-3b (BD Biosciences, San Diego, Calif.). The assay was performed according to the manufacturer's instructions. The expression of cytokines was analyzed and quantified with Multi-Analyst software (BioRad, Munich, Germany). Data are expressed as percentage expression of the housekeeping gene GAPDH.

Cytokine release

Cytokine release from splenocytes or TIL was measured after 48 or 72 h after stimulation with irradiated B16-melanoma cells. Interferon-γ (IFN-γ) and IL-4 production were measured by sandwich enzyme-linked immunoassay (ELISA) using OptEIA kits (BD Biosciences, San Diego, Calif.), following the manufacturer's instructions.

Statistical analysis

The statistical significance of data was determined by the Mann–Whitney-*U*-test. The level of significance was set at $P < 0.05$.

Results

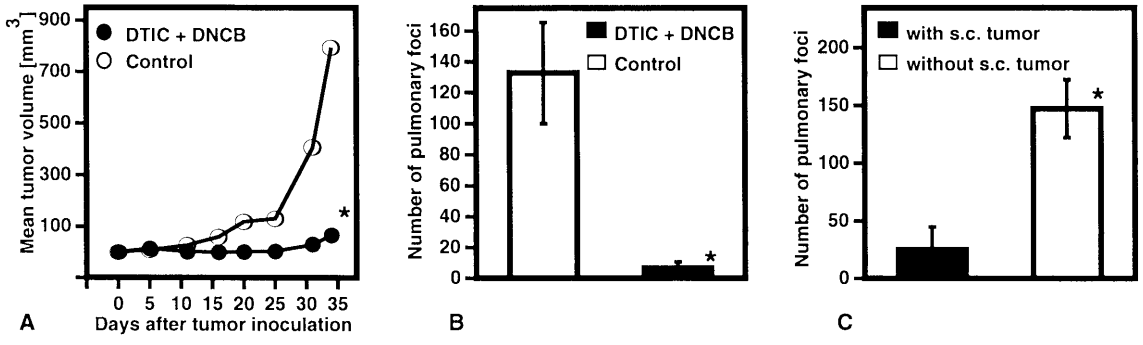
Effect of chemoimmunotherapy with DTIC and DNCB on s.c. tumors and pulmonary metastases

Using the B16-melanoma model, we initially confirmed that chemoimmunotherapy combining the epifocal application of DNCB on cutaneous metastases with the systemic administration of DTIC resulted in a significantly delayed growth of s.c. tumors in syngeneic C57BL/6 mice (Fig. 1A). In 5 out of 7 mice the s.c. tumor regressed completely in response to DTIC/DNCB treatment, and did not reoccur within the observation period of the experiment. The remaining 2 mice and untreated control animals suffered from progressing s.c. tumors (data not shown). Next, we analyzed the effect of this therapy on pulmonary metastases. Seven days after inoculation of s.c. tumors, pulmonary metastases were induced by injection of B16-melanoma cells into the lateral tail vein. Treatment of s.c. tumors with DTIC and DNCB started 2 days later and also resulted in a significant reduction in the numbers of metastatic foci on the lung surface (Fig. 1B). Pulmonary foci were reduced from 133 ± 31 in untreated control mice to 7 ± 4 in treated mice. Further experiments have shown that the therapeutic effect on experimentally induced pulmonary metastases at least partially depends on the presence of an s.c. tumor. After treatment with DTIC and DNCB, 25 ± 20 pulmonary metastases were detected in mice with an s.c. tumor, whereas 147 ± 25 were found in mice without an induced s.c. tumor (Fig. 1C).

Induction of an anti-tumorigenic immune response by treatment with DTIC and DNCB

Spontaneous regression of primary melanoma as well as infiltration of the tumor with immunocompetent cells,

Fig. 1. **A** Effect of DTIC/DNCB on the growth of s.c. tumors. C57BL/6 mice were injected with 2×10^6 B16 cells on day 0. Seven days thereafter, mice were treated with DTIC (50 mg/kg i.p.). One day later tumors were coated with DNCB (2%). This schedule was repeated on days 12, 16, and 20 (using DNCB 1%). Tumor volume over time is given as means of different treatment groups ($n = 7$). **B** Decreased number of pulmonary foci after treatment of s.c. tumor with DTIC and DNCB. Seven days after inoculation of s.c. tumors, a single-cell suspension of 2×10^6 tumor cells was injected into the lateral tail vein. Treatment (see above) of s.c. tumors started 48 h later. Mice ($n = 7$) received 7 treatment cycles, one every 4th day. Surface lung metastases were counted under a dissecting microscope 30 days after inoculation. **C** The experimental procedure was the same as that described in **B**, but in one group of mice no s.c. tumors were induced. These animals received DNCB on their backs. The data are representative of the results of three (**A**, **B**) or two (**C**) independent replicate experiments. $^*P < 0.05$



i.e. macrophages and T cells, are taken as evidence of an ongoing immunological reaction against the tumor [1, 6, 54]. Thus, we addressed the question of whether this chemoimmunotherapy was capable of eliciting a specific antitumor immune response. To this end, splenic lymphocytes from treated and untreated mice were isolated and assessed in a 4-h ^{51}Cr-release assay for their cytolytic activity against B16-melanoma cells. As depicted in Fig. 2A, splenic lymphocytes obtained from treated mice displayed three-fold higher specific cytolytic activity against B16-melanoma cells than those derived from control animals. When syngeneic EL-4 cells were used as target cells, no killing was observed, indicating specificity of the observed cytotoxic activity against melanoma cells (Fig. 2B). Lytic activity against B16-melanoma cells was also detectable in tumor-bearing control mice, indicating that the presence of the tumor alone might be sufficient to induce to some extent an anti-tumorigenic response. Next, we analyzed the release of IFN-γ from splenic lymphocytes cocultured with irradiated B16-melanoma cells. The amount of IFN-γ was more than 8 times higher in samples obtained from mice treated with DTIC and DNCB than in those from untreated control animals (Fig. 2C). In contrast, no IL-4 was detectable (data not shown). Taken together, these data indicate that chemoimmunotherapy with DTIC and DNCB results in a systemic anti-tumorigenic effect.

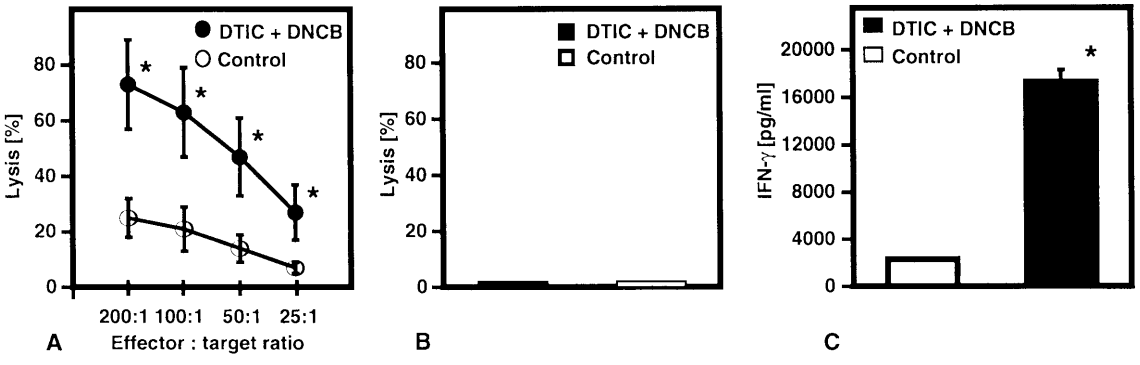
The role of different T cell subpopulations

Since it has been shown that different T cell subpopulations can be involved in eliciting an anti-tumorigenic response, we investigated their role in the observed antitumor activity induced by DTIC/DNCB treatment. Therefore, T cell subpopulations ($CD4^+$, $CD8^+$) and natural killer (NK) cells were isolated from splenocytes by positive magnetic separation and tested for their lytic activity against B16-melanoma cells or the NK-sensitive YAC-1 lymphoma cells. Both $CD4^+$ as well as $CD8^+$ T cells from treated mice showed significantly higher specific killing of B16 cells than those derived from control mice (Fig. 3). In contrast, NK cell activity was not influenced by treatment with DTIC and DNCB, indicating that the antitumorigenic effect of this therapy is not mediated via NK cells.

Effect of chemoimmunotherapy on TIL

It is well known that melanoma is associated with a chronic inflammatory infiltrate, composed predominantly of lymphocytes. This observation provided the opportunity to investigate the local immunologic reaction against the tumor. Therefore, TIL from s.c. B16 tumors were isolated and assessed for their capability to lyse syngeneic B16-melanoma cells. TIL derived from tumors of animals treated with DTIC and DNCB (2 cycles) displayed significant higher cytolytic activity against B16-melanoma cells than those from untreated control animals (Fig. 4A). We also analyzed the release of cytokines from TIL after in vitro restimulation with irradiated B16-melanoma cells. The amount of IFN-γ was found to be higher in samples from treated mice than in those from controls (Fig. 4B). IL-4 was not detectable (data not shown). These data indicate that chemoimmunotherapy with DTIC and DNCB influences the local anti-tumorigenic response. A possible mode of action could be the generation of a suitable cytokine environment facilitating this response. This prompted us to examine the expression of cytokine RNA in s.c. tumors using the RNase protection assay. The RNA levels of IFN-γ and TNF-α were found to be significantly elevated (two-fold) after treatment with DTIC and DNCB when compared to control animals (Fig. 5). The observed increase in IL-6 RNA expression was not significant. IL-2 and IL-4 expression was not detectable using this assay. However, reverse transcription–polymerase chain reaction (RT–PCR) analysis

Fig. 2. **A** Presence of specific cytotoxic splenic lymphocytes. The percentage of lysed B16-melanoma cells by splenic lymphocytes was measured at different E:T ratios in a ^{51}Cr-release assay. Effector cells were isolated subsequent to treatment with DTIC and DNCB (7 cycles) and restimulated in vitro. After 3 days of stimulation, cells were recovered and analyzed for their cytolytic activity against B16-melanoma cells. **B** As a control the lytic activity against syngeneic EL-4 cells was determined at an E:T ratio of 200:1. **C** Splenic lymphocytes from treated (7 cycles) and untreated mice were cocultured in the presence of irradiated B16-melanoma cells. The supernatants were harvested after 48 h and the levels of IFN-γ were determined by cytokine-specific ELISA. The data are expressed as the mean ± SD of triplicate cultures and are representative of three experiments. $*P < 0.05$



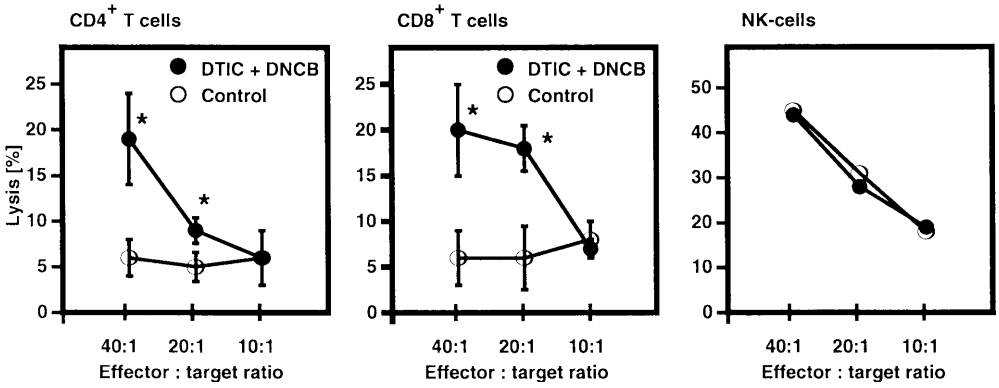


Fig. 3. Percentage of lysed B16-melanoma cells or YAC-1 lymphoma cells by different T cell subpopulations or NK cells was measured at indicated E:T ratios in a ^{51}Cr-release assay. Effector cells were isolated subsequent to treatment with DTIC and DNCB (7 cycles) and restimulated in vitro. After 3 days of stimulation, different T cell subpopulations were purified by immunomagnetic separation and analyzed for their cytolytic activity against B16-melanoma cells ($CD4^+$ and $CD8^+$ T cells) or YAC-1 lymphoma cells (NK cells). The data are expressed as the mean ± SD of triplicate cultures and are representative of three experiments. $^*P < 0.05$

revealed that IL-2 was expressed in s.c. tumors while IL-4 was not (data not shown).

Effect of contact sensitizers and skin irritants on the growth of s.c. tumors

The enhanced cytolytic activity of splenic lymphocytes and TIL against B16-melanoma cells after treatment with DTIC and DNCB suggests that T cells are involved in the anti-tumorigenic response elicited by this therapy. Contact sensitizers elicit a specific T cell-mediated immune response, whereas skin irritants induce a non-specific inflammation which on histological examination may be indistingishable from allergic contact dermatitis. We examined whether therapy with DTIC in combination with skin irritants instead of contact sensitizers was equally effective (Fig. 6). Treatment with DTIC in combination with the contact sensitizers DNCB (1%) or oxazolone (1.5%) resulted in a significantly delayed growth of s.c. tumors. In contrast, when DNCB was replaced by the skin irritants croton oil (2%) or tributyltin (600 nmol) no effect on the tumor was observed, although visible skin inflammation occurred, indicating that a T cell-dependent immune reaction is necessary for an efficient anti-tumorigenic response.

In order to further support the relevance of T cells in this response, the therapeutic effect was examined in immunodeficient mice. B16-melanoma cells were injected s.c. into T cell-deficient C57BL/6 RAG-$1^{-/-}$ mice and therapy with DTIC and DNCB (4 cycles) was started 7 days thereafter. As depicted in Fig. 7, in the absence of T cells no therapeutic effect on s.c. tumors could be observed. This observation further suggests a T cell-dependent mechanism for the demonstrated therapeutic effect.

Discussion

In a previous study we reported the efficacy of chemoimmunotherapy with DTIC and DNCB against B16-melanoma, leading to decreased growth of both s.c. tumors and pulmonary metastases in a syngeneic

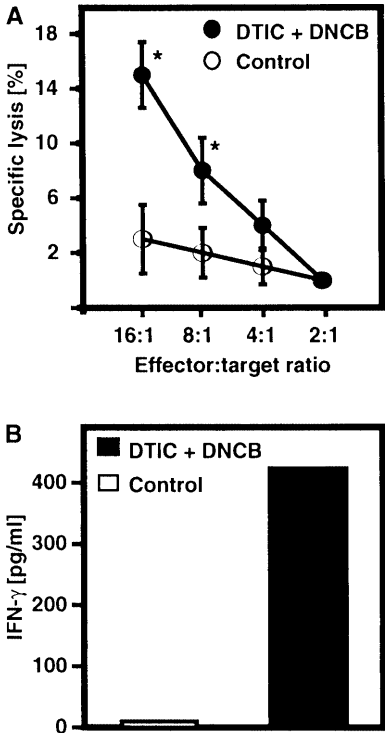


Fig. 4. **A** Lysis of B16-melanoma cells by tumor-infiltrating lymphocytes. TIL were isolated from s.c. tumors of treated (2 cycles of DTIC/DNCB treatment) or untreated C57BL/6 mice by immunomagnetic separation. After 3 days of in vitro restimulation, the cytolytic activity of TIL against B16-melanoma cells was determined at the indicated E:T ratios using a microcytotoxicity assay. The data are expressed as the mean ± SD of triplicate cultures and are representative of three experiments. $^*P < 0.05$. **B** Purified TIL from treated (2 cycles of DTIC/DNCB treatment) and untreated control animals were cocultured in the presence of irradiated B16-melanoma cells. The supernatants were harvested after 48 h and the levels of IFN-γ were determined by cytokine-specific ELISA. The data are expressed as the mean of duplicate cultures and are representative of two experiments

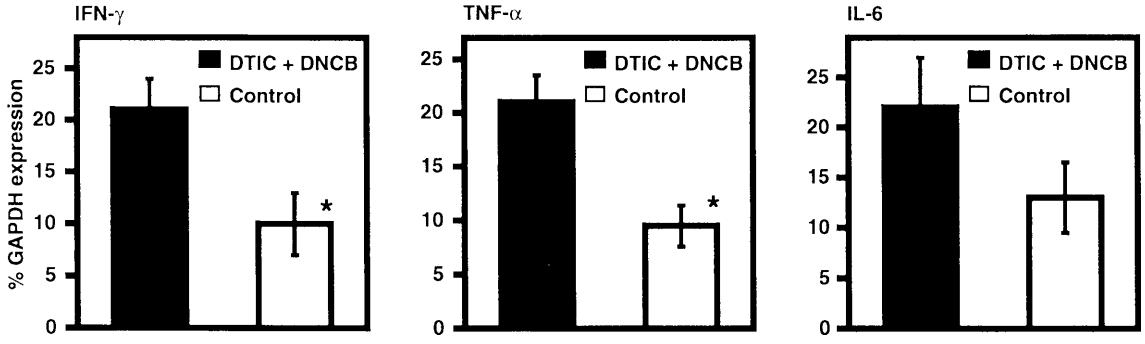


Fig. 5. Analysis of cytokine mRNA expression by RNase protection assay. Total RNA was isolated from s.c. tumors of treated (2 cycles of DTIC/DNCB treatment) and untreated mice. For each hybridization reaction, 10 μg of total RNA was used. The expression of cytokine levels was analyzed and quantified with Multi-Analyst software (BioRad, Munich, Germany). Data are expressed as percentage expression of the housekeeping gene GAPDH and as the mean ± SD of triplicate determinations. Data are representative of two experiments. $*P < 0.05$

tumor model [61]. In the present study, we have demonstrated that this therapy elicts a specific T cell-dependent immune response against the tumor. Treatment of tumor-bearing mice resulted in an increased cytotoxic activity of splenic lymphocytes against B16-melanoma cells. Analysis of different T cell subpopulations revealed that both $CD4^+$ and $CD8^+$ T cells have the capability to lyse B16-melanoma cells. Several studies using adoptively transferred purified T cell subsets or in vivo depletion studies have firmly established an important role for tumor-specific $CD8^+$ cytotoxic T lymphocytes (CTL) in antitumor immunity [32]. $CD8^+$ CTL are able to lyse tumor cells directly upon recognition of antigen-MHC class I complexes expressed by the tumor, and their ability to eradicate tumors in vivo has been demonstrated. The role of $CD4^+$ T cells has received far less attention. However, the contribution of tumor-specific $CD4^+$ T cells to the development of an effective antitumor response has been demonstrated in different studies [16, 23, 24, 57]. The requirement for $CD4^+$ T cells has been mainly attributed to providing help for the generation of a fully activated tumor-reactive CTL [11, 20, 21, 31, 39, 40]. In addition, several lines of evidence suggest that $CD4^+$ T cells play a more diversified role in the antitumor immune response, including several distinct effector functions. Previous studies have reported a lytic activity of melanoma-specific $CD4^+$ T cells [29, 52, 53, 63]. $CD4^+$ T cells may act by a either direct mechanism against MHC class II-positive tumors or by an indirect mechanism against MHC class II-negative tumors. Studies by Hung et al. have proposed that $CD4^+$ T cells may eliminate tumors by activation and recruitment of effector cells, including macrophages and eosinophils [23]. Other studies have suggested that

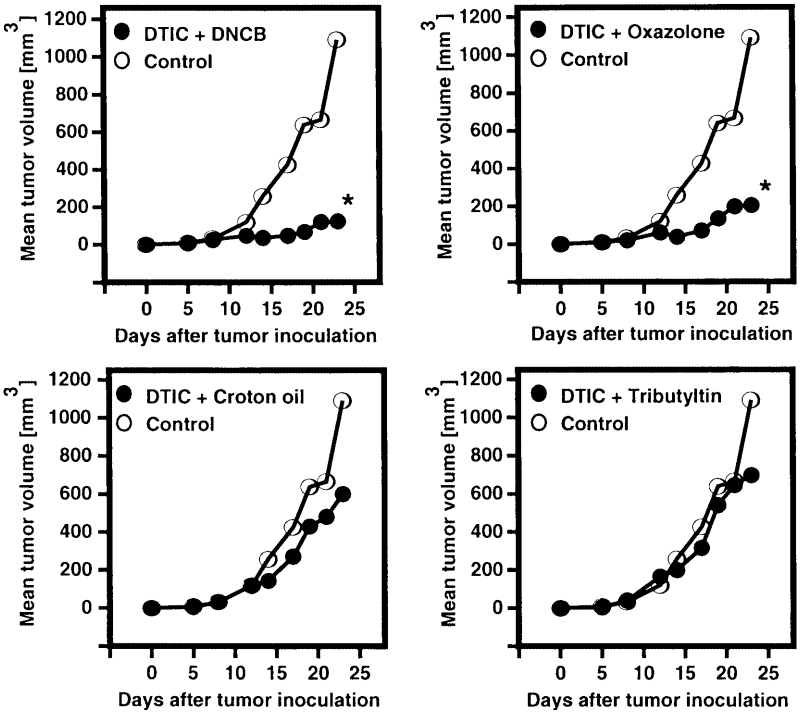


Fig. 6. Effect of contact sensitizers and skin irritants on the growth of s.c. tumors. C57BL/6 mice were injected with 2×10^6 B16 cells on day 0. Seven days thereafter mice were treated with DTIC (50 mg/kg, i.p.); 24 h later, tumors were coated with the contact sensitizers DNCB (1%) or oxazolone (1.5%) or with the skin irritants croton oil (2%) or tributyltin (600 nmol). This schedule was repeated on days 12, 16, and 20. Compounds were dissolved in actone and olive oil (4:1). Tumor volume over time is given as means of different treatment groups ($n = 7$). $*P < 0.05$

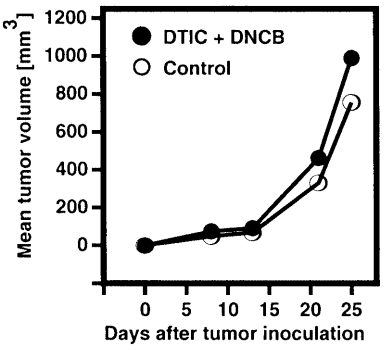


Fig. 7. Effect of DTIC/DNCB on the growth of s.c. tumors in immunodeficient mice. Female C57BL/6 RAG-1$^{-/-}$ mice were injected s.c. with 2×10^6 B16 cells on day 0. Seven days thereafter, mice were treated with DTIC (50 mg/kg, i.p.). One day later tumors were coated with DNCB (2%). This schedule was repeated on days 12, 16, and 20 (using DNCB 1%). Tumor volume over time is given as means of different treatment groups ($n=7$)

cytokines such as IFN-γ that are secreted by CD4$^+$ T cells might be involved in antitumor activities [35]. Whether such effector mechanisms are involved in animals treated with DTIC and DNCB is unknown. However, our findings strongly suggest a T cell-dependent mechanism for the observed therapeutic effect. First, the therapeutic effect achieved by treatment with DTIC in combination with contact sensitizers could not be observed when contact sensitizers were replaced by skin irritants which elicit a non-specific inflammation. The used doses of contact sensitizers and irritants have been found to induce an increase in ear thickness in the mouse ear swelling test. A second line of evidence indicating the requirement of T cells was provided by studies in T-cell deficient RAG-1$^{-/-}$ mice where combined DTIC and DNCB therapy was ineffective.

Contact hypersensitivity is a T cell-mediated immune response of the skin occurring in sensitized individuals that come in contact with the sensitizing hapten [9, 19, 49]. During sensitization, DNCB is covalently coupled to cell surface proteins. Langerhans cells, the dendritic cells of the epidermis, capture the hapten, enter the lymphatic vessels, and migrate to skin-draining lymph nodes where hapten–MHC complexes are presented to naive T cells. Subsequent challenge with the same contact sensitizer induces recruitment of the primed T cells to the challenge site. Since the contact sensitizer DNCB is applied in close vicinity to the s.c. tumor, it is tempting to speculate that DNCB induces antigen-presenting cells such as Langerhans cells to develop the capability to capture and present tumor-derived antigens, inducing a T-cell response to melanoma. However, when tumor-bearing mice were treated with DNCB alone, no therapeutic effect was observed, indicating that DTIC and DNCB act in a synergistic mode against the tumor. Initially it was of concern whether cytotoxic drugs would impair immune-mediated antitumor responses. However, it has been shown that DTIC reduces neither the IL-2-mediated induction of cytotoxic effector cells nor the induction of secondary cytokines [26, 43]. Furthermore, we have observed that DNCB-induced contact hypersensitivity is not influenced by treatment with DTIC [61].

Our data demonstrate that treatment of s.c. tumors with DTIC and DNCB results in increased mRNA levels for IFN-γ, TNF-α, and IL-6, suggesting that treatment of tumor-bearing mice results in the production of cytokines in the tumor microenvironment which may modulate tumor–host interactions. In fact, the lytic activity against B16-melanoma cells from TIL derived from treated animals was significantly higher than from those of untreated control animals. However, it is also possible that the observed increase in cytokine production simply reflects the status of activation of effector cells infiltrating the tumor. Nevertheless, the important role of cytokines in modulationg immune responses against melanoma as well as other tumors has been demonstrated by several studies [27, 30, 33, 36, 62]. Recently, different groups have shown an association between Th1-like cytokine mRNA expression and spontaneously regressing melanomas. Lowes et al. observed increased mRNA levels of IL-2, TNF-β, and IFN-γ in regressing melanomas compared to non-regressing melanomas [27]. In contrast, no differences were found in Th2-like cytokines. Wagner et al. found an association between regressing melanoma and increased mRNA expression of IL-2, granulocyte–macrophage colony-stimulating factor (GM–CSF), and IL-15 [62]. Recently, it has been demonstrated that antibody-mediated targeting of IL-2 or lymphotoxin-α to the tumor microenvironment mounts an effective cellular response against murine melanoma [3, 47, 55]. In addition, Huang et al. have shown that vaccination of C57BL/6 mice with irradiated B16-melanoma cells, genetically modified to release GM–CSF, induced immunity against B16 tumors, possibly due to the ability of the locally produced GM–CSF to activate antigen-presenting cells during the priming phase to process and present tumor antigens to both CD4$^+$ and CD8$^+$ T cells [22].

One strategy against malignant neoplasms is the use of chemotherapeutic agents in combination with immunotherapy. The rationale for this combination is based on the finding that chemotherapeutic agents may potentiate immune responses to the tumor. Several studies have provided evidence for the increased antigenicity of tumors after exposure to cytotoxic drugs. Therefore, enhancing the intrinsic immunogenicity of tumors may be an important modality to increase the effectiveness of immunotherapy. It has been reported that treatment of tumor cells with DTIC increases the immunogenicity of these cells by inducing new antigenic specificities that are not found on parental cells [4, 5, 38]. Since DTIC is an alkylating agent it is likely that the induction of mutations could lead to the expression of new antigens, which are then recognized by effector cells. This idea is supported by the observation that single point mutations can cause strong immunogenicity [34, 42] Pieper et al. identified a melanoma antigen recognized by CD4$^+$ TIL. A C to T transition mutation causing a threonine to isoleucine replacement in

triosephosphate isomerase (TPI) created a neoepitope whose T cell-stimulatory activity was enhanced by 5 logs compared with the wild type [42]. It is conceivable that the therapeutic effect observed in our tumor model partly depends on such a mechanism. This would be in line with the following observation. When B16-melanoma cells were pretreated for 48 h with temozolomide, the active metabolite of DTIC, before they were used as target cells in ^{51}Cr-release assays, the lytic activity of splenic lymphocytes derived from DTIC/DNCB-treated mice was increased two-fold compared to untreated B16 cells (data not shown). Alternatively, it is possible that cell death induced by cytotoxic agents may increase the immunogenicity of the tumor. In fact, dendritic cells which acquired antigen from apoptotic cells are able to induce MHC class I-restricted cytotoxic T cells and antitumor immunity [13, 17, 46].

The results of this report demonstrate that the efficacy of chemoimmunotherapy with DTIC and DNCB in the treatment of malignant melanoma in the B16-tumor model depends on T cells. This therapeutic approach induced a specific immune response which is likely due to either the priming of naive T cells, the activation of cytotoxic effector cells, or both. It is unknown whether the mechanisms observed in this model also occur in human melanoma. Trcka et al. reported an increased expression of HLA-DR and IL-2 receptors on peripheral T cells from a patient responding to therapy with DTIC and DNCB. In addition, IFN-γ was detectable in the skin metastases of some patients [58]. These data suggest that T cells are important for therapeutic efficacy. However, it remains to be determined by further studies whether this therapy results in the generation of melanoma specific T cells in human melanoma.

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