Primary Chemically Induced Tumors Induce Profound Immunosuppression Concomitant with Apoptosis and Alterations in Signal Transduction in T Cells and NK Cells1

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ABSTRACT

Whereas transplantable tumors can be readily cured with immunotherapeutic approaches, similar therapies in cancer patients have been less effective. This difference may be explained by an immunosuppressive result from the presence of a slowly growing primary tumor in the patient, whereas the immune system in a mouse with a rapidly proliferating transplantable tumor would be less affected. As a more appropriate model to the immune dysfunction in patients, slowly progressing primary tumors were induced by the carcinogen methylcholanthrene (MC) in mice. Their ability to induce immunosuppression in T cells and natural killer (NK) cells was compared to that of rapidly growing transplanted MC-induced tumors. The results demonstrate that mice bearing primary MC tumors had significantly diminished T-cell and NK-cell functions, impaired capacity to produce Th1 cytokines, and markedly reduced levels of the signal-transducing ζ chain in T cells and NK cells, similar to that described in cancer patients. Moreover, a substantial number of CD8+ T cells in mice with large primary MC tumors were undergoing apoptosis, correlating with alterations in CD4/CD8 ratios. In contrast, T cells and NK cells from mice bearing rapidly growing transplanted tumors were only marginally affected. These findings could explain the apparent discrepancy between the consistent findings of a diminished immune response and alterations in signal transduction in cancer patients as compared to the less reproducible observations in murine transplantable tumors. In addition, they could explain the differences in the high efficacy of immunotherapy in mice with transplantable tumors and the low therapeutic results in cancer patients.

INTRODUCTION

Patients with advanced cancer and mice with large transplantable tumors have a compromised immune system with decreased delayed-type hypersensitivity responses, and they exhibit alterations in the proliferative and cytotoxic capacities of their T cells and NK3 cells and in host cytokine expression and production (1–5). Recent observations showing alterations in signal-transducing molecules in T and NK cells from tumor-bearing mice and cancer patients provide a molecular basis to better understand this immune dysfunction. The original observation in mice bearing a slowly growing mouse colon carcinoma (5) showed an altered pattern of protein tyrosine phosphorylation, a reduction of the protein tyrosine kinases p56lck and p59fyn, and a decreased CD3 ζ chain. These findings have subsequently been confirmed and extended to a variety of human tumors, including renal, colorectal, ovarian, liver, gastric, pancreatic, and cervical carcinomas and melanomas (6–11). Of particular interest is the correlation observed between ζ expression and the disease stage in cancer patients (8–13). Although the reproducibility of these findings in patients has been consistent, they have been more difficult to reproduce in murine models using transplantable tumors (14, 15).

Experimental evaluations of new immunotherapeutic strategies are almost exclusively based on models of transplantable mouse tumors, where it has been possible to cure established tumors using cytokine-secreting tumors, tumor-derived antigenic peptides with various types of adjuvants, activated killer cells, or cytokines (16). In contrast, a smaller proportion of the patients have shown responses to similar therapeutic approaches (17). These apparently contradictory observations between transplantable murine tumor models and cancer patients could be explained in part by the ability of a slowly progressing primary tumor to induce an immune dysfunction as compared to a rapidly growing transplantable murine tumor, which might spare the immune response.

Therefore, primary MC induced sarcomas and rapidly growing transplantable MC sarcomas were compared with regard to their ability to induce immunosuppression and alterations of signal-transducing molecules in B6 mice. Results demonstrate that mice bearing primary tumors have a severely compromised immune response, with T cells and NK cells showing decreased cytotoxic and proliferative capacity and with reduced levels of Th1 cytokine production and with spontaneous apoptosis of their CD8+ T cells and markedly reduced levels of the CD3 ζ chain. In contrast, the expression of the same molecule and the function of the T cells and NK cells in animals carrying a transplanted rapidly growing tumor was only marginally affected. These findings may provide a possible explanation to the marked differences in therapeutic efficacy of immunotherapy observed between cancer patients and experimental mice bearing transplanted tumors.

MATERIALS AND METHODS

Tumors. Adult B6 mice, bred and maintained at the animal facilities of the Microbiology and Tumor Biology Center at the Karolinska Institute, were injected once i.m. in the flank with 0.5 mg of MC (Sigma Chemical Co., St. Louis, MO) dissolved in 0.05 ml of corn oil. Mice were inspected weekly, and tumors were measured weekly with a calimeter. The primary MC tumors were passaged once, here defined as “1st-generation tumor,” or several times, defined as “>1st generation” by s.c. inoculation into B6 mice. Eight different primary MC tumors were transplanted as 1st generation tumors, and 12 different MC tumors were serially transplanted as >1st generation tumors. The original tumor was also always maintained frozen.

In some experiments, the long-term serially transplanted MC57X tumor (18) of B6 origin was used.

Generation of Influenza Virus-specific CTLs. Influenza virus-specific CTLs were generated by in vivo priming and in vitro restimulation. Briefly,
mice were injected with 200 hemagglutination units of influenza virus strain PR8 diluted in PBS. Ten days later, spleens were harvested, and 20 × 10^6 single cells were restimulated with equal numbers of X-irradiated (2000 rad) PR8-infected syngeneic spleen cells in 10 ml of complete medium. After 5 days, cultures were tested for the ability to lyse the influenza NP366 peptide (ASNENMETM)-pulsed or nonpulsed ⁵¹Cr-labeled RMA-S targets. Target cells were prepared by culturing cells for 1 h at 37°C in 5% CO₂ plus 150 μCi of Na₂¹⁵CrO₄ (Amersham, Amersham, England). They were washed four times, and 5000 peptide-pulsed (3 μg/ml) or nonpulsed ⁵¹Cr-labeled target cells per well were mixed with effector lymphocytes to yield several E:T cell ratios. After 5 h of incubation, 75 μl of supernatant were harvested from each well and counted in a gamma counter. Specific lysis was calculated according to the formula: percentage specific lysis = 100 × (experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm). All assays were done in triplicate; spontaneous release and percent error never exceeded 20 and 15%, respectively.

NK Assays. A 4-h standard ⁵¹Cr release assay was used to measure NK cell susceptibility. Briefly, spleens were harvested, and single cells were used fresh as NK effector cells. Target cells were prepared by culturing cells for 1 h at 37°C in 5% CO₂ plus 150 μCi of Na₂¹⁵CrO₄ (Amersham) They were washed four times, and 5000 ⁵¹Cr-labeled target cells per well were mixed with effector cells to yield several E:T cell ratios. After 4 h of incubation, 75 μl of supernatant were harvested from each well and counted in a gamma counter. Specific lysis was calculated according to the formula: percentage specific lysis = 100 × (experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm). All assays were done in triplicate; spontaneous release and percent error never exceeded 20 and 15%, respectively.

Analysis of Signal Transduction Molecules. The expression of CD3 ζ was studied by flow cytometry analysis of permeabilized cells, as described previously (12). Briefly, mouse spleen cells were fixed with 0.5% formaldehyde (Sigma) in PBS for 30 min on ice, permeabilized with digitonin (10 μg/ml; Sigma) for 10 min on ice. Cells were then incubated with FITC-conjugated antimonkey ζ chain mAb (Oncozeta 1, kindly provided by Biomira USA, Cranbury, NJ) as well as with phycoerythrin-conjugated anti-CD4, anti-CD8, or anti-CD3 (PharMingen) and then subjected to flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson). The percentage of T cells was evaluated by analyzing the number of CD4⁺ plus CD8⁺ cells or by the number of CD3⁺-positive cells.

Cytokine Testing. TNF content was determined by testing the cytotoxic effect on Wehi 164 clone 13 cells in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (19). IFN-γ was determined by using commercially available matched antibody pairs for mouse cytokines ELISA (ImmunoKontact, Bioggio, Switzerland, and BioSource, Fleurus, Belgium, respectively) according to the manufacturers’ recommendations.

Apoptosis Analysis. All procedures were performed using a flow cytometry based method according to the manufacturer’s protocol (Annexin V kit; Becton Dickinson). Cells undergoing apoptosis were defined as those positive for Annexin V and negative for the vital dye 7-amino-actinomycin D (Sigma).

**RESULTS**

**Expression of Signal-transducing ζ Chains in T Cells and NK Cells during Growth of Primary MC Sarcomas.** A total of 40 primary tumors were induced by i.m. injection of MC in adult B6 mice, which resulted in i.m. growth of tumors to a large size (>15 mm in diameter) after ~16 weeks (15.5 ± 1.9) in all mice injected. T cells derived from splenocytes of individual B6 mice bearing large (>15 mm in diameter), intermediate (5–15 mm), or small (<5 mm) primary tumors or from animals injected with MC but without detectable tumors were analyzed by flow cytometry and Western blot for the expression of ζ chain. T cells from mice with large MC tumors expressed, on average, 40% of ζ chain compared to normal age- and sex-matched control mice (Table 1 and Fig. 1A). T cells from mice with tumors of intermediate size also expressed significantly less ζ than controls (P < 0.001), although they expressed more than those with large tumors. The decreased expression of ζ chain was also demonstrated by Western blot analysis of T cells from mice with primary MC tumors (data not shown). None of the 24 mice bearing large or intermediate primary tumors had ζ expression within the range of the 54 normal control mice (Fig. 1A). Even T cells from mice with small primary tumors had a significantly (P = 0.008) decreased ζ chain expression as compared to mice without tumors. Mice that had been injected with MC but had not yet developed tumors did not show a decreased expression of ζ (P = 0.289), demonstrating that the effect was induced by the tumor and not by the chemical carcinogen peripheral blood-derived T cells demonstrated a decrease in CD3 ζ staining, which was comparable to that observed in the splenic T cells (Fig. 2, B and A, respectively). The expression of ζ was suppressed to the same extent in the CD4⁻ as compared to the CD8⁻ T cells (data not shown). Furthermore, NK1.1⁺ cells from mice bearing large or intermediate MC primary tumors also showed a decreased expression of ζ chain (Fig. 1B, P < 0.001).

In contrast, cell surface expression of CD3ε was not significantly altered in spleen cells of mice with progressively growing primary MC tumors (MFI = 133 ± 35, n = 20 for control mice versus MFI = 125 ± 32, n = 10 for mice with large tumors; P = 0.64), nor was there a significant change in the expression of cell surface located NK1.1, CD4 or CD8 molecules (data not shown).

**Expression of Signal-transducing ζ Chains in T Cells and NK Cells during Growth of Transplantable MC Sarcomas.** A single-cell suspension of the primary MC tumors was transplanted into syngeneic B6 mice and called “1st generation” transplantable MC tumors. These transplantable MC tumors, in contrast to the primary MC tumors, developed rapidly (3.5 ± 2 weeks) into large (>15 mm in diameter) tumors or from animals injected with MC but without detectable tumors. The transplanted MC tumors, in contrast to the primary MC tumors, developed rapidly (3.5 ± 2 weeks) into large (>15 mm in diameter) tumors or from animals injected with MC but without detectable tumors.
immunosuppression in mice with chemically induced tumors

1st generation tumor previously been passed as transplantable tumors (CD16z generation) had only a slightly reduced
Table 1). T cells from mice with serially passaged MC tumors (data not shown) were analyzed in spleens from primary (Fig. 3A) or transplantable (data not shown) tumors, there was an increase in the CD4/CD8 ratio in tumor-bearing mice that was significant even in mice with small tumors. The CD4/CD8 ratio in MC-treated non-tumor-bearing mice was, however, unaltered (Fig. 3).

Interestingly, the increase in CD4/CD8 ratio observed in mice with large primary MC tumors correlated (r = 0.84) with significant levels of spontaneous apoptosis observed in the CD8+ T cells (data not shown) concurrently with a decreased proportion of CD3ε+ T cells (Table 1). When the proportion of CD4+ and CD8+ T cells were analyzed in spleens from primary (Fig. 3A) or transplantable (data not shown) tumors, there was an increase in the CD4/CD8 ratio in tumor-bearing mice that was significant even in mice with small tumors. The CD4/CD8 ratio in MC-treated non-tumor-bearing mice was, however, unaltered (Fig. 3).

Loss of Influenza-specific CD8+ CTL and of NK Cytotoxicity in Spleen Cells from Mice with Primary and Transplantable MC Tumors. We next tested whether progressively growing tumors influenced cytotoxic T-cell functions. Spleen cells from tumor-bearing, influenza virus-infected mice were restimulated once in vitro with the virus and then tested against RMA-S target cells pulsed with the influenza NP366 peptide. The CTL activity was markedly reduced
when generated from spleens derived from mice with primary MC tumors, as compared to sex- and age-matched controls (Fig. 4A).

CTL activity was also suppressed in mice bearing large 1st generation transplantable MC tumors, although less than in mice with primary tumors (Fig. 4B). CTLs from tumor-free MC treated mice, or from mice with serially transplantable (>1st generation) tumors of small or intermediate size generated CTLs that were as efficient as those from controls. The observed reduction in CTL activity generated from spleens of primary MC tumors is probably not the result of the lower number of T cells in these because the progressive growth of serially transplantable tumors induced a comparable reduction in the proportion of T cells in the spleen, while having no or little effect on the generation of CTL function (Table 1).

NK cytotoxicity against the YAC-1 target also showed a progressive decline in splenocytes from mice with primary MC tumors and large 1st generation transplantable tumors (Fig. 5A). In contrast, mice with tumors passaged during several generations had an intact NK activity (Fig. 5B), although sometimes a small but significant reduction in NK activity of these mice was also observed (data not shown).

Loss of Anti-CD3-induced Proliferation and Th1 Cytokine Production in Spleen Cells from Mice with Primary and Transplantable MC Tumors. T-cell proliferation assays following stimulation with anti-CD3 mAb were performed (Fig. 6A), in which T cells from mice with primary MC induced tumors of large or intermediate size responded less efficiently (n = 12, P < 0.001) and n = 8, P < 0.001 respectively) as compared to control mice (n = 22). T cells from mice (n = 16) bearing large 1st generation transplantable tumors also had a small but significantly (P = 0.023) decreased proliferative response, whereas those with large serially passaged tumors had T cells with an intact (P = 0.29) proliferative response.

TNF (Fig. 6B) and IFN-γ (Fig. 6C) production was measured following anti-CD3 stimulation. Spleen cells from mice with large primary MC induced tumors were almost totally devoid of the capacity to produce IFN-γ or TNF in response to anti-CD3 stimulation. The ability to respond by Th1 cytokine production as a result of anti-CD3 stimulation decreased proportionally to the size of the tumor, but mice carrying tumors of small or intermediate size also produced significantly (P < 0.001) less of these two cytokines. Spleen cells from mice that had been treated with MC but had not yet developed tumors produced unaltered or almost unaltered amounts of IFN-γ and TNF compared to control spleens (P = 0.47 for TNF and P = 0.05 for IFN-γ).

In contrast, spleen cells from mice with progressively growing large transplantable MC tumors that had been serially transplanted for >1st generation had unaltered capacity to produce IFN-γ and TNF following anti-CD3 stimulation (n = 9, P = 0.8 as compared to control mice, n = 20; Fig. 6). Mice bearing 1st generation transplantable MC tumors had spleen cells which were weakly although significantly (n = 9, P < 0.001) impaired in their capacity to produce Th1 cytokines in response to anti-CD3 stimulation, in comparison to control mice (Fig. 6).

Linear regression analysis, in which the titers of IFN-γ and TNF or proliferation indexes for spleens of individual mice were plotted against the ζ values in the CD3+ population, revealed a strong correlation coefficient for production of IFN-γ (n = 70, r = 0.92), TNF (n = 70, r = 0.86), and T-cell proliferation indexes (n = 81, r = 0.81; Fig. 7).

We, therefore, conclude that the T-cell proliferative responses as well as the capacity to produce the Th1 cytokines IFN-γ and TNF are severely impaired in spleen cells from mice with primary MC-induced sarcomas and also significantly, although much less so, in mice with 1st generation transplantable MC sarcomas. Furthermore, the production of Th1 cytokines and the proliferative response correlate with decreased expression of the signal-transducing ζ molecules.

DISCUSSION

Alterations in T-cell functions and signal transduction molecules have been observed in patients with several types of cancer (20). However, these findings have been less reproducible in mice bearing transplantable tumors that have been passaged multiple times (14, 15). Here, we report that primary mouse sarcomas induce dramatic changes in immune functions and signal transduction molecules in both T and NK cells that occur concurrently with deficient cytotoxic and proliferative functions and diminished capacity to produce Th1 cytokines. The alteration seen in CD3 ζ expression was detectable in T cells from peripheral blood and spleen and involved all tested subsets of lymphocytes expressing the ζ chain, as demonstrated previously in cancer patients (8, 12). The suppressed ζ expression and the decreased CTL and NK cytotoxicity in spleen cells from mice bearing primary MC tumors was not the result of the known immunosuppres-

Fig. 3. Alterations in CD4/CD8 ratios and in spontaneous apoptosis in the CD8+ T cell subset of mice with primary MC tumors. A, spleen cells from mice which had no palpable tumor (no tumor) or mice with small (Small tumor <5 mm), intermediate (Interm. tumor 5–15 mm), or large (Large tumor >15 mm) tumors from MC-treated groups were analyzed for CD4 and CD8 expression by flow cytometry, and the CD4/CD8 ratio was calculated. Data points: X, values from spleen cells of individually analyzed mice; bars, means (bars, SD). The number of mice in each group (n) is shown. Statistically significant Ps are indicated: **, <1%; *, <5%; ns, >10%. B, linear regression analysis of the percentage of apoptotic cells versus the CD4/CD8 ratio of the same spleen cell population in TB mice and control mice. The CD8+ spleen cells derived from tumor-free (control) B6 mice (∗) or from tumor-bearing (TB) mice with primary MC tumors (∗) were analyzed by flow cytometry for apoptosis, defined as cells positive for Annexin V and negative for 7-amino-actinomycin D.
sive effects of MC (21, 22) because MC-treated but tumor-free mice were unaffected.

Previous observations in cancer patients showed a correlation between the stage of the disease and the expression of CD3 $\zeta$ for different types of tumors (8–13). The decrease in CD3 $\zeta$ expression seen in mice with primary tumors also appeared to parallel the tumor burden. In contrast, large serially transplanted tumors only marginally affected the immune response and signal transduction molecules. Mice with primary tumors also had severely reduced capacity to generate influenza-specific CTLs and had very low NK activity as well as decreased proliferative T-cell response and diminished production of IFN-$\gamma$ and TNF. Patients with cervical cancer or melanomas were also found to have deficient influenza-specific CTL responses (23, 24) and diminished capacity to produce TNF-$\alpha$ correlating with decreased expression of CD3 $\zeta$ (10). In our study, the reduction in specific CTLs and NK activity and in T cell proliferation and cytokine production observed in mice with transplantable 1st generation tumors was, in some experiments, strong and approached that observed in mice with primary tumors of the same size. In contrast, in serially passaged MC tumors (>1st generation), only a very small or no suppression was observed, and these mice only had a marginal decrease of CD3 $\zeta$ expression in T cells and no changes in CD16 $\zeta$ expression. Levey and Srivastava (15) have found that T cells from mice bearing large serially transplanted tumors appeared to express normal signal transduction molecules, although mice with very advanced disease sometimes had a decreased $\zeta$ chain expression. Their study included four different chemically and UV-induced long-term established serially transplanted tumors, which would be comparable to the serially transplanted (>1st generation) MC tumors in our study. Therefore, the data observed in serially transplanted tumors

\[ \text{Fig. 4. CTL responses generated from mice with primary or transplantable MC tumors.} \]

\[ \text{Influenza virus-specific CTLs were generated by } \textit{in vivo} \text{ priming (influenza RP8 virus) and } \textit{in vitro} \text{ restimulation (influenza NP366 peptide) of spleen cells derived from tumor free untreated or MC-treated mice or from mice with transplantable or primary MC tumors of the sizes indicated. The CTLs were tested against influenza peptide-pulsed RMA-S cells (-----) or nonpulsed RMA-S cells (----). Results from two independent experiments are shown in A and B.} \]

\[ \text{Fig. 5. NK responses from mice with primary or transplantable MC tumors. Spleen cells derived from tumor free untreated or MC-treated mice or from mice with transplantable or primary MC tumors of the sizes indicated were used as effector cells against the YAC-1 target cell. Results from two independent experiments are shown in A and B.} \]

\[ \text{Fig. 6. Proliferative T cell responses and cytokine production generated from anti-CD3-stimulated spleen cells of mice with primary or transplantable MC tumors. Spleen cells from groups of mice which had been treated with MC or inoculated with MC tumors, according to the same protocol as used in Fig. 1, were analyzed for anti-CD3 mAb-induced T-cell proliferation (A) and for the production of the cytokines TNF (B) and IFN-$\gamma$ (C), as described in "Materials and Methods." The number of mice in each group is indicated in parentheses. Columns, means from individually analyzed mouse spleens; bars, SD.} \]
in this study agree with their observations. The growth characteristics of certain slowly growing transplantable tumors, including the MCA-38 colon carcinoma and the CMS5 sarcoma (5, 25), may be more similar to that observed for primary MC tumors in this study because they can induce similar types of alterations in signal-transducing molecules. In line with this, alterations in CD3 z chain expression in mice bearing primary MC tumors went largely in parallel with a decrease in the percentage of splenic T cells and an increased CD4/CD8 ratio. The decreased proportion of T cells may simply be a "dilution" effect due to a large influx of other cell types, such as macrophages and granulocytes. Alternatively, T cells and NK cells may be actively deleted by apoptosis induced by FasL interaction, expressed by the tumor cells (28), or by H2O2 generated by activated macrophages (29, 30).

In agreement with this, we found spontaneous apoptosis in the CD8+ T cell subset of primary MC tumor-bearing mice. Apoptosis in T cells and NK cells of tumor-bearing mice may, therefore, be related to their poor functional capacity and also to their decreased expression of signal-transducing molecules. In line with this, alterations in CD3 z chain expression by coincubation with ovarian carcinoma cells were suggested to be related to apoptosis induced by FasL expression on tumor cells (31). Also, apoptosis was shown to be induced in NK cells in vitro by H2O2 produced by macrophages (29). Therefore, apoptosis observed by us in tumor-bearing mice may have been induced directly by H2O2 (30) or could be an indirect consequence of the capacity of reactive oxygen species to induce CD95 ligand (FasL) or CD95 (Fas) expression in T cells (32, 33). One interesting possibility would therefore be if the observed alteration in z expression in MC tumors may represent a "preapoptotic," although reversible (12), condition of T cells or NK cells.

Proteolytic degradation of mouse splenocyte z and p56 lck by granulocyte-associated cathepsin G can occur in the spleen cell lysate when analyzing the expression of these molecules by biochemical methods (34). This is unlikely the case in this report because we could observe a decreased expression of z, even when spleen cells from tumor-bearing mice were immediately fixed by mincing the spleen cells directly in 0.5% formaldehyde (e.g., see experiment in Fig. 2), thus inactivating proteolytic enzymes, or when purified T cells devoid of granulocytes were analyzed both by flow cytometry (Fig. 2) or Western blot (data not shown). However, extreme care should be taken to rapidly fix cells prior to the further time consuming processing for flow cytometry analysis because artifactual alterations of z can be observed with time ex vivo (data not shown).

Our findings might help explain why mice with transplantable tumors can be consistently cured by immunotherapeutic interventions because their immune system is relatively intact even with a large tumor burden. One important implication from these results is that it would be more comparable to the clinical setting to test immunotherapeutic modalities in mice with primary induced tumors because their compromised immune system is more comparable to what has been described in cancer patients with advanced disease. Research aimed at developing drugs that can counteract suppression of antitumor activity may benefit from using the primary MC tumor model. These drugs should, when given in combination with immunotherapy, provide new and promising avenues for the treatment of cancer.

ACKNOWLEDGMENTS

The excellent technical assistance of Maj-Lis Solberg and Margareta Hage- lin at the animal facility of Microbiology and Tumor Biology Center is gratefully acknowledged.
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