IgG2a Induced by Interleukin (IL) 12-producing Tumor Cell Vaccines but not IgG1 Induced by IL-4 Vaccine Is Associated with the Eradication of Experimental Metastases

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ABSTRACT

We evaluated whether antibody response correlates with tumor therapy by cytokine gene-modified tumor cell vaccines. To characterize the antibody (Ab) response against a known antigen, colon carcinoma C26 cells and C26 variants engineered to produce interleukin (IL) 12 or IL-4 were further transduced to express the human tumor-associated antigen gp38 folate receptor (FR) or Fr. Irradiated IL-12- and IL-4-producing C26/FRα cells vaccinated 50 and 30% of mice bearing C26/FRα lung micrometastases. Treatment induced a rapid, CD4-dependent Ab production dominated by IgG2a and IgG1 in response to the IL-12 or IL-4 vaccine, respectively. In contrast, untreated tumor-bearing mice showed a late serological response dominated by IgM. Anti-FRα IgG1 and IgG2a were able to suppress tumor metastesases upon passive transfer in vivo. Sera from mice cured by the IL-12 vaccine displayed a higher binding activity, a higher anti-FRα IgG2a content, and a higher complement-mediated tumor cell lysis in vitro compared to the sera from nonresponder mice. Such a correlation was not found in the sera of mice treated with the IL-4 vaccine. These data indicate that cytokine-producing tumor cell vaccines strongly influence antibody response, and that in the case of the IL-12-based vaccine, the Ab titer correlates with the therapeutic response, thus suggesting its use for monitoring the outcome of vaccination in cancer patients.

INTRODUCTION

There is increasing evidence for both humoral and cellular immune responses to human TAAs1 (1, 2). The recent development of the so-called SEREX technique (1), which allows the use of patient serum to screen a cDNA library from the autologous tumor, often resulted in the detection of novel TAAs. Among these TAAs, there are antigens that have already been identified by CTL recognition (3). In addition, it has been formally demonstrated that strong humoral and cellular immune responses against a defined TAA can coexist in the same patient (4).

However, the significance of antitumor antibodies in cancer patients is not univocal, and their role in tumor rejection is still controversial. In cancer patients, the presence of an Ab to the tumor antigens has been correlated with either a poor or good prognosis in different tumor types or even in the same tumor types (5). In murine models, B cells have been shown to participate in tumor eradication processes (6, 7) and in antitumor memory responses (8), whereas they have also been found to inhibit tumor rejection upon adoptive transfer in allogeneic recipients (9) and to interfere with the induction of tumor immunity (10).

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3 The abbreviations used are: TAA, tumor-associated antigen; IL, interleukin; FR, folate receptor; ADC, antibody-dependent cellular cytotoxicity; Ab, antibody; mAb, monoclonal Ab; NK, natural killer, Th, T helper.

An association between the development of an antitumor Ab response and prolonged survival was observed after vaccinating melanoma patients with gangiosides or cell vaccines (11, 12). In mice, vaccination with homologous xenogeneic protein or protein expressed in insect cell induced an Ab to Tyrosinase-related-protein autoantigen, mediating melanoma rejection (13, 14).

Most of the ongoing cancer vaccine trials are designed to stimulate antitumor T-cell immunity (15). Cell-based vaccines are largely tested clinically (16). Cytokine gene-modified tumor vaccines elicit a massive local infiltration, resulting in T-cell activation and tumor rejection in preclinical studies (17). Whether an antitumor Ab response is activated by cytokine gene-modified tumor vaccines in a therapeutic setting and whether such a response has a role in tumor elimination have not been studied.

We have previously shown that the therapeutic efficacy of IL-12-producing tumor vaccine is associated with the production of an Ab that is able to bind complement (18). In the present study, we have tested whether the Ab response induced by cytokine-producing tumor vaccines may correlate with tumor eradication. To this purpose, the human TAA FRα (19) was used as a known target antigen upon transduction into C26 carcinoma cells. The serological response induced in mice bearing C26/FRα lung metastases by tumor vaccines engineered to produce IL-4 or IL-12, the major cytokines influencing T-cell-dependent Ab responses (20), has been studied. Immunotherapy by vaccinations with IL-4- or IL-12-producing C26/FRα cells cured 30–50% of mice bearing C26/FRα lung metastases. To test whether an antitumor Ab response predicts the therapeutic outcome in response to tumor cell vaccines, we have studied the anti-FRα Ab response in mice that were cured or not cured by vaccinations.

MATERIALS AND METHODS

mAbs and Fluorescence-activated Cell-sorting Analysis. Murine mAbs MO18 (IgG1) and MO19 (IgG2a) directed to nonoverlapping epitopes of the FRα (19, 21), which is overexpressed in about 90% of human ovarian carcinomas, were used for in vivo and in vitro experiments. Murine mAbs MINT5 (IgG1) and MGR6 (IgG2a), which recognize epidermal growth factor receptor and HER-2/neu, respectively (22, 23), were used as irrelevant isotype-matched controls in the in vivo experiments. mAb MO18 was kindly provided in purified form by Centocor (Malvern, PA), whereas the other mAbs were purified from mouse ascitic fluid or culture supernatants by affinity chromatography on insolubilized protein A (Pierce, Rockford, IL) according to their isotype. GK1.5 (ATCC TIB 207) and 2.43 (TIB 210) rat IgG2b mAbs used for in vivo CD4 and CD8 T-cell depletion were purified from ascites by using a commercial kit (E-Z-SEP; Pharmacia Biotech). To detect tumor-reactive immunoglobulin, indirect immunofluorescence was performed as described previously (21) by using FITC goat anti-mouse Ig (Technogenetics, Milan, Italy) or FITC rat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, and IgE mAbs (PharMingen, San Diego, CA) to determine the immunoglobulin isotype. Staining was analyzed by flow cytometric analysis.

Cell Lines. C26/FRα, C26/IL-4/FRα, and C26/IL-12/FRα cells producing murine IL-4 or IL-12 and expressing human FRα were obtained by retroviral transduction as described previously (8, 24). The FRα vector was constructed by cloning a 910-bp EcoRI fragment of the human gene into retroviral vector.

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**RESULTS**

**Vaccinations with IL-12- or IL-4-producing C26/FRα Cells Eradicate C26/FRα Lung Micrometastases.** To evaluate whether vaccination with cytokine-transduced tumor cells had therapeutic activity, mice bearing C26/FRα lung metastases were given s.c. injections of C26/IL-12/FRα or C26/IL-4/FRα irradiated cells and monitored for survival. The results showed that immunotherapy by vaccinations with IL-12- or IL-4-producing C26/FRα cells cured 56 and 36% of the mice, respectively (Fig. 1). Survivors were free of disease (data not shown). Parental C26/FRα cells used as vaccines were not effective (data not shown), indicating that cytokine expression was necessary to improve the immunogenicity of the vaccine.

**Antitumor Ab Response in Tumor Bearers Is Strongly Influenced by Treatment with IL-12- or IL-4-producing Tumor Vaccines.** The presence of antitumor Ab was tested in the sera of C26/FRα tumor-bearing mice that were untreated or treated with the C26/IL-12/FRα or C26/IL-4/FRα vaccines. Sera collected at various time points when mice showed no evidence of disease were evaluated for the ability to stain C26/FRα cells. High levels of tumor-reactive Ab were detected in the sera of vaccinated tumor-bearing mice but not in untreated tumor-bearing mice on day 13 and in vaccinated and untreated mice at subsequent time points (Fig. 2), indicating that vaccination induced a faster antitumor humoral response than that which spontaneously occurred in tumor-bearing mice.

Both IL-12 (Fig. 3) and IL-4 (data not shown) vaccines induced Abs binding the human ovarian carcinoma IGROV-1 cells overexpressing FRα and the FRα-transduced C26 cells, but not the human MeWo melanoma cells used as negative control. A weak staining of parental C26 cells was also observed, which was likely due to the Ab response to undefined endogenous C26 antigens (18). Our study then focused on the Ab response to the FRα antigen induced by vaccinations in mice bearing C26/FRα lung metastases.
The Ab response was abrogated in CD4-depleted but not in CD8-depleted mice, thus indicating that the anti-FRa Ab response induced by the IL-12 and IL-4 vaccines was T-cell dependent (Fig. 4).

To compare the Ab titers induced by the two types of vaccines, titration experiments were performed. The IGROV-1 cell staining assay showed that sera diluted at 1:200 and 1:400 from mice treated with the IL-12 vaccine yielded higher mean fluorescence values than sera from mice treated with the IL-4 vaccine (data not shown). Binding assays by 125I-labeled anti-mouse Ig revealed high levels of anti-FRa Ab in vaccinated mice; the IL-12 vaccine induced a stronger response than the IL-4 vaccine (Fig. 5).

We then analyzed the immunoglobulin isotypes of anti-FRa Abs in the sera obtained from vaccinated mice. Significant differences in the levels of G1, G2a, and M anti-FRa immunoglobulin subtypes were detected; high levels of IgG1 were shown after vaccination with C26/IL-4/FRa cells, whereas IgG2a was the predominant isotype after vaccination with C26/IL-12/FRa cells, and IgM was the most represented subclass in the sera from nonvaccinated tumor bearers (Fig. 6).

Altogether, these data indicate that immunotherapy using IL-12- or IL-4-producing tumor vaccines strongly deflected the antitumor Ab response by inducing the isotype switch to IgG2a and IgG1, respectively. In addition, as demonstrated by binding assays (Fig. 5), IL-12 seemed to be a stronger adjuvant for Ab response than IL-4.

Anti-FRa mAbs of Both IgG1 and IgG2a Subclasses Suppress Tumor Growth in Vivo. To test whether the anti-FRa Ab of the IgG1 and IgG2a subclasses has a different capacity to inhibit C26/FRa tumor growth in vivo, we took advantage of the availability of mouse mAbs of matching isotypes and specificity produced by the hybridomas MOV18 and MOV19, respectively (21). Previous studies have shown that the treatment of metastases by passive humoral immunotherapy requires repeated injections of mAbs given at high doses (150–600 μg; Refs. 13, 30, and 31). This contrasts with the low amount of sera that can be obtained from mice and with the need to purify and quantitate its activity. Therefore, to compare the in vivo antitumor activity of the anti-FRa IgG2a and IgG1 subclasses, purified mAbs MOV19 and MOV18 were passively transferred into tumor-bearing mice, and the rejection of lung metastases was evaluated. As shown in Fig. 7, both anti-FRa mAbs determined a marked reduction of lung metastases (a median of 22 and 17 metastases, respectively, versus 250 metastases in untreated controls). Treatment with isotype-matched mouse mAbs against hEGFR and HER-2/neu was ineffective against C26/FRa metastases that did not express these Ags.

Thus, IgG2a and IgG1 have a potential role in the mechanism of eradication of tumor metastases induced by treatment with IL-12- and IL-4-producing tumor cell vaccines.

Evaluation of Ab Response in Mice Responding or Not Responding to Immunotherapy using IL-12 and IL-4 Vaccines. We analyzed the sera of mice that were cured (responders) or not cured (nonresponders) by IL-12 or IL-4 vaccines to test whether the magnitude of Ab response correlated with the therapeutic effect. Individual samples of sera obtained after vaccination were stored and tested after survival follow-up, and the sera from responders and nonresponders were individuated, pooled separately, and tested for binding and functional activity.
HUMORAL AND ANTITUMOR RESPONSES TO TUMOR VACCINES IN MICE

Sera obtained from mice cured by IL-12 vaccination [responders (R)] showed a higher binding to IGROV-1 cells than sera from nonresponder (N) mice (Fig. 8A). In addition, the level of IgG2a was significantly higher in the sera from R mice than in that from N mice (Fig. 8C). This difference was further confirmed at the functional level by testing the complement-dependent tumor cytotoxicity in vitro (Fig. 8E). These data clearly indicate that the antitumor IgG2a response induced by IL-12 vaccines correlated with treatment efficacy.

However, the same analysis carried out with the sera from mice vaccinated with IL-4-producing cells failed to show a correlation between therapeutic activity and tumor binding (Fig. 8B). IgG1 content (Fig. 8D), and in vitro cytotoxicity by ADCC (Fig. 8F).

DISCUSSION

We studied the Ab response induced by cytokine gene-transduced tumor vaccines in a therapeutic setting using a preclinical model. The results indicate that both the IL-12 and IL-4 vaccines induced a fast humoral response against a defined TAA transduced into the target tumor. Furthermore, the Ab titers measured after IL-12 vaccination were correlated with the clinical response.

IL-12 has been shown to be a potent adjuvant for humoral immunity, increasing serum IgG2a levels as well as IgG2b and IgG3 levels and suppressing IgG1 and IgE responses in different model systems (32–34). IL-12 acts through the induction of IFN-γ production, supporting Th1 cell differentiation (35) with specific help for IgG2a responses (36), and through IFN-γ-independent mechanisms (37). In several systems, IL-12 and IL-4 have been found to have opposite effects on humoral immunity and to direct the immune response toward Th1 or Th2 predominance. IL-4 favors the growth of Th2 cells, promotes the differentiation of stem cells to B cells, and stimulates their growth and membrane receptor expression. IL-4 is an isotype switch factor favoring IgG1 and IgE in mouse and IgG1 in man (20). Percile et al. (8) have shown that multiple vaccinations with tumor cells engineered to release IL-4 resulted in the production of antitumor IgG1 Ab as well as IgE and IgA. After IL-4 vaccination, we detected FRα-specific IgG1 but did not detect other immunoglobulin subtypes. Transforming growth factor β produced by C26 cells (data not shown) might inhibit IL-4-induced IgE switching (20).

Antitumor Ab can mediate tumor destruction through the classical immune mechanisms of ADCC and complement fixation or by impairing cancer cell growth by blocking growth factor receptors. In murine studies, tumor eradication by the passive transfer of mAbs occurs through either ADCC or complement fixation, as shown for the eradication of B16 melanoma (13, 30, 31). Neutralization of the complement system by cobra venom abrogated the antitumor effect of IgG2a mAb (13), whereas an in vivo depletion of NK cells by pretreatment with Ab directed against asialo-GM1 and NK1.1 abrogated the effect of IgG2b mAbs, suggesting that ADCC was responsible for tumor destruction (30, 31).

An Ab-based mechanism of immunity to SV40-induced tumors in BALB/c mice was found to be mediated by ADCC (38). We have found that although both IgG1 MOV18 and IgG2a MOV19 mouse mAbs activate ADCC-mediated tumor cytotoxicity, the IgG1 isotype induced a stronger cytolyis (Fig. 8E). In keeping with published data (8), we found that fresh splenic lymphocytes from IL-4–vaccinated mice were more lytic in ADCC assays than cells obtained from naive mice. Although we have not studied this phenomenon, it is likely that...
the IL-4 vaccine determines the activation/proliferation of Fc+ cells in the splenic population. In the mouse system, IgG2a mediates ADCC via the higher affinity type I Fc Receptor (CD64), whereas IgG1 mediates ADCC through interaction with the type II (CD32) and type III (CD16) Fc Receptor in macrophages, monocytes, neutrophils, and NK effector cells (39). IgG1 may also participate in tumor destruction by mediating eosinophil degranulation (40). Eosinophil is commonly found to infiltrate IL-4-transduced tumors and can take part in the destruction of metastases induced by the IL-4 vaccine through IL-4 and IL-5 released in situ by specific T cells. In addition, antitumor Ab may influence specific T-cell responses by modulating antigen processing as demonstrated by Smitse et al. (41).

Additive effects of antitumor Ab and CD8 immunity have been demonstrated in a mouse lymphoma (42). In this tumor model, immunotherapy by IL-12 and IL-4 vaccines activates a strong antitumor T-cell response, and tumor eradication was shown to be CD8 dependent, a finding common to other murine models (43, 44). When CTLs activated by the IL-12 vaccine were studied in relation to survival, no difference in their bulk cytotoxic activity against C26/Fox tumor was shown, but rather different Ag specificities that could be related to tumor immunoselection and escape were shown.5 Although the role of Ab response has not been directly tested in B-cell knockout mice, our data clearly indicate that the passive transfer of Abs with the same specificity and isotype of those induced by vaccination can reduce lung metastases.

We have evaluated whether measurement of serological response is a useful indicator for immunological follow-up after vaccination and whether such a parameter can predict the therapeutic outcome. High IgM and low IgG titers in patients’ serum had the strongest association with overall survival in melanoma patients treated with vaccinations of a polyvalent allogeneic melanoma cell vaccine (45). However, few studies have assessed the induction of antigen-specific antitumor Ab response induced by tumor cell vaccines in man (46), and we are not aware of any published study dealing with the relevance of antigen-specific Ab responses in a therapeutic setting. The efficacy of the vaccination on the generation of immune response is evaluated by comparing immunological parameters before and after treatment; however, which parameter is correlated with clinical response remains unknown (47). Our retrospective analysis of Ab response in vaccinated mice was carried out to address this issue. Because vaccinations induced high-titered immunoglobulin G, which is dependent on help from CD4 T cells, IgG levels can be a measure of specific T-cell activation. Our experiments showed that IgG2a is indeed correlated with tumor eradication induced by the IL-12 vaccine, whereas IgG1 induced by vaccination with IL-4-producing cells is not. The lack of correlation between the IgG1 level and the antitumor effect remains to be studied. We are currently investigating the mechanism underlying the curative effect of the IL-4 vaccine. Preliminary results indicate that the therapeutic effect is CD8 dependent, and that CD8 T cells activated by vaccination display a type 2 phenotype of cytokine production (IL-4, IL-5, and IL-10) and are not cytotoxic but can eradicate metastases by interacting with granulocytes (48). Because most of the ongoing vaccination studies are based on cytokines inducing a Th1 response, and because an immunological parameter predictive of the therapeutic outcome has not yet been identified, our results suggest the measurement of antitumor IgG2a titers during the follow-up of vaccinated patients as an indicator of the host immune response.

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