Differential Interleukin 12 Responsiveness for Interferon γ Production in Advanced Stages of Cancer Patients Correlates with Performance Status

Kazuko Uno, Junko Setoguchi, Mari Tanigawa, Atsuko Kishi, Makoto Ogawa, Hideo Saotome, Hiromi Fujiwara, and Tsunataro Kishida
Louis Pasteur Center for Medical Research, Kyoto 606, Japan [K. U., J. S., M. T., A. K., T. K.]; Biomedical Research Center, Osaka University Medical School, Osaka 565, Japan [M. O., H. F.]; and Genetics Institute, Inc., Cambridge, Massachusetts 02140 [H. S.]

INTRODUCTION
IL-122 has been shown to exhibit pleiotropic effects on T cells and NK cells (reviewed in Refs. 1 and 2). This cytokine enhances NK (3) and CTL (4) activities; acts as a NK and T-cell growth factor (5–7); stimulates the secretion of various cytokines, particularly that of IFN-γ by NK and T cells (3, 8); and promotes maturation of the Th1 helper T-cell subset (1, 9).

The therapeutic activity of IL-12 has been observed in various murine tumor models (10–13). Moreover, it has recently been shown that systemic administration of IL-12 induces complete tumor regression through a series of immune responses by the host (reviewed in Refs. 14 and 15): IL-12 strikingly stimulates antitumor T cells and NK cells to produce high levels of an effector cytokine, IFN-γ (11, 12), and allows these reactivated T cells to infiltrate tumor sites (16) and produce IFN-γ that plays a critical role in tumor regression (17). In tumor-bearing individuals, IFN-γ production alone is not sufficient to induce significant therapeutic effects (18). Nevertheless, this initial process is critical for the IL-12-mediated antitumor effect because treatment with anti-IFN-γ monoclonal antibody before IL-12 therapy abrogates the antitumor efficacy of IL-12 (11, 12).

The tumor-bearing state is a representative pathophysiological condition in which abnormal cytokine production is induced and by which T-cell responses are modulated (reviewed in Ref. 19). Whereas clinical trials of IL-12 have just started in the United States, Europe, and Japan, a fundamental issue remains to be resolved regarding whether IL-12 can similarly stimulate T cells and NK cells from various patients in various states of tumor-induced immunomodulation (20). In the present study, we assessed the IL-12 responsiveness of PBMCs from various cancer patients by stimulating whole blood itself with rIL-12 and measuring IFN-γ production by PBMCs contained in whole blood. The results show that a remarkable difference exists in IL-12 responsiveness among cancer patients. The differential IL-12 reactivity correlates with neither the type of tumors nor the number of PBMCs present in whole blood but rather with the PS of either patients at all cancer stages or those at a given advanced stage. These results provide an important implication in evaluating the IL-12 responsiveness of cancer patients in ongoing clinical trials of IL-12 immunotherapy.

PATIENTS AND METHODS
Subjects. In this investigation, various cancer-bearing patients and healthy subjects were selected from those who were...
receiving medical care or a health check with blood tests at the polyclinic of Louis Pasteur Center for Medical Research (Kyoto, Japan). Blood was drawn after informed consent. The healthy subjects had no acute infection and no history of chronic infectious or autoimmune disease and exhibited no abnormal value on routine blood tests. The cancer patient group consisted of patients with hepatic tumors \( n = 29 \), stomach tumors \( n = 9 \), colon tumors \( n = 24 \), lung tumors \( n = 19 \), breast tumors \( n = 9 \), ovarian tumors \( n = 3 \), uterine tumors \( n = 3 \), and other types of tumors \( n = 13 \) and included 50 men and 59 women (mean age, 58.1 ± 12.4 years; range, 25–88 years). Patients who had received radiotherapy or chemotherapy within the past 2 months were excluded.

**Cytokines.** rIL-12 was supplied by Genetics Institute, Inc. (Cambridge, MA). Human leukocyte IFN-\( \gamma \) (natural form) was supplied by Nihon Chemicals Research Co., Ltd. (Hyogo, Japan) and used as the standard. The titer of IFN was standardized to NIH reference Gg23-901-530 and expressed in International Units per milliliter.

**Preparation of Blood Samples and Isolation of PBMCs.** Venous blood was drawn using an evacuated blood collection tube containing sodium heparin. To isolate PBMCs, heparinized venous blood was drawn using a VACUTAINER CPT cell preparation tube with sodium heparin (Becton Dickinson, San Jose, CA). PBMCs were recovered from the upper layers after centrifugation at 3000 rpm for 20 min and washed with MEM. PBMCs were suspended at \( 10^6 \) cells/ml in RPMI 1640 supplemented with FCS, 5 mM HEPES, and antibiotics.

**Stimulation of Whole Blood or PBMCs with rIL-12.** Heparinized venous blood was diluted 1:4 with MEM. Stimulation of whole blood with rIL-12 was performed according to the procedure described previously as the whole blood method (21): diluted blood was cultured with various concentrations of rIL-12 in F-sptz tubes (Eiken Kizai Co., Ltd., Tokyo, Japan). For stimulation of PBMCs, isolated PBMCs were suspended in RPMI 1640 and distributed to each well of 48-well NUNCLON culture plates (Nalge NUNC Intern., Roskilde, Denmark) at a volume of 1 ml/well. Cultures were conducted in a CO\(_2\) incubator (5% CO\(_2\)) at 37°C for 20 h. SNs were harvested by centrifugation at 3000 rpm and stored at −80°C until use.

**Measurement of IFN-\( \gamma \) Concentration.** IFN-\( \gamma \) concentrations were measured by ELISA. The human IFN-\( \gamma \) ELISA system was prepared using two types of anti-human IFN-\( \gamma \) monoclonal antibody 2G1 [2G1 was purchased from Endogen (Cambridge, MA)] and biotinylated B133.5 (B133.5 was purchased from Endogen and biotinylated in our laboratory) as well as human IFN-\( \gamma \). One IU/ml in our ELISA system corresponded to approximately 0.1 unit/ml in Pharmingen ELISA kits (recombinant human IFN-\( \gamma \); specific activity, 6.7 \( \times \) 10\(^6\) units/mg). Human TNF-\( \alpha \) kits (Genzyme Corp., Cambridge, MA) were used for the determination of TNF-\( \alpha \) concentration.

**Statistical Analysis.** Results are presented as the arithmetic mean ± SE. Statistical analysis was performed using ANOVA and Fisher's protected least significant difference. Statistical calculations were computed with StatView software (Abacus Concepts, Inc., Berkeley, CA).
Concentrations of rIL-12 used for stimulation (pg/ml).

Fig. 3 Comparison of IL-12-stimulated IFN-γ production between healthy individuals and cancer patients. Diluted whole blood samples from healthy individuals and cancer patients were cultured with various concentrations of rIL-12 for 20 h in F-spitz tubes, and culture SNs were assayed for IFN-γ concentrations.

Healthy individuals

Cancer patients

healthy individuals. Because some samples from both groups of positive responders exhibited somewhat lower responses on stimulation with 10000 pg/ml IL-12 compared to those obtained after stimulation with 1000 pg/ml IL-12, the following analyses were done by stimulating whole blood with 1000 pg/ml rIL-12.

A comparison was made between controls (n = 30) and various cancer-bearing patients (n = 27) for IFN-γ production by whole blood after stimulation with 1000 pg/ml IL-12 (Fig. 3). IL-12-induced IFN-γ production in the cancer patients (3.2 ± 0.73 IU/ml) was significantly lower than that seen in the healthy individuals (7.9 ± 1.3 IU/ml; P < 0.005). The control group showed a wide variation in IFN-γ production, and the difference in the mean values of IFN-γ production between the two groups may be due in part to the presence of healthy individuals exhibiting a particularly high IFN-γ production. Nevertheless, more importantly, all 30 of the healthy individuals exhibited positive responses (more than 1.5 IU/ml), whereas approximately half of the cancer patients showed almost-null responses.

Correlation of IL-12 Responsiveness in Cancer Patients with PS. IL-12 responsiveness is considered to be modulated by various factors and/or conditions in cancer patients. We investigated which variable(s) is responsible for producing differential IL-12 responsiveness. These variables included cancer stages, PS at the time of examination, the number of lymphocytes as responders to IL-12, and the type of tumors. Cancer patients were graded as one of three different cancer stages (stages I + II, III, and IV) according to the tumor-node-metastasis (TNM) classification of Union Internationale Contre Cancer (UICC). IL-12-stimulated IFN-γ production in whole blood from cancer patients at various stages is summarized in Fig. 4. The mean IFN-γ production values of stage I + II, stage III, and stage IV patients were 4.4 ± 0.8, 10.5 ± 4.5, and 2.7 ± 0.7 IU/ml, respectively. Patients at stage IV showed a lower capacity for IFN-γ production than did those at stage III (< 0.05). A high mean value in stage III and a statistical difference between stage III and stage IV may be related to the exceptionally high IL-12 responsiveness of one patient in stage III. Thus, it is obscure whether there is actually a difference between cancer stages. In fact, although patients exhibiting null responses were more frequent at stage IV than at earlier stages, it was also obvious that more than half of the stage IV patients showed positive (low to considerable) responses.

Instead of the classification based on cancer stages, we classified all patients in Fig. 4 as one of three different PS grades (PS 0, PS 1 + 2, and PS 3 + 4) according to the description (22) by the American Joint Committee on Cancer (Fig. 5). The results show that a reduced capacity of IFN-γ production in the patients was observed as their PS status progressed. The mean values of IFN-γ production are as follows: healthy individuals,
Differential IL-12 Responsiveness in Cancer Patients

Remarkable that the incidence of patients with null or marginal responses increases from PS 0 (20.5%) through PS 1 + 2 (34.1%) to PS 3 + 4 (85%). This was also the case when stage IV patients only were classified according to the three grades of PS (Fig. 6); the mean values of IFN-γ production were 4.8 ± 3.7 IU/ml in PS 0 patients, 2.7 ± 0.6 IU/ml in PS 1 + 2 patients, and 0.3 ± 0.9 IU/ml in PS 3 + 4 patients (PS 0 patients versus PS 3 + 4 patients, P < 0.05), and the incidence of null responses increased with PS grading. Taken collectively, these data indicate that IL-12 responsiveness differs among cancer patients, and differential responsiveness correlates with PS.

Relationship of IL-12 Responsiveness to the Number of Lymphocytes in the Blood and to the Type of Tumors. To determine whether IL-12 responsiveness is related to the number of lymphocytes contained in whole blood, we determined both the IFN-γ-producing capacity and the number of lymphocytes in each PS group (Fig. 7). Although the mean number of lymphocytes decreased with PS grading, there was no significant difference between even PS 0 patients and PS 3 + 4 patients. Thus, the reduction of IL-12 responsiveness in PS 3 + 4 patients does not depend on the decrease in the number of lymphocytes as responders to IL-12.

We also compared the IFN-γ-producing capacity in patients bearing different types of tumors. As shown in Fig. 8, there was no significant difference in IL-12 responsiveness among these patients. The results show that IL-12 responsiveness decreases with PS grade in each categorized tumor group.

IL-12 Responsiveness of PBMCs Isolated from Blood Samples Showing Null/Marginal Responses. We examined whether PBMCs contained in blood samples showing null/marginal responses have the capacity to respond to IL-12 when isolated from the blood and stimulated with IL-12 in the absence of plasma. PBMCs were isolated from the blood of 13 patients (PS 1 + 2) exhibiting zero to only marginal levels (<1.0 IU/ml) of IFN-γ production by whole blood. PBMCs suspended in RPMI 1640 were stimulated with 1000 pg/ml rIL-12. As shown in Fig. 9, 7 of 13 PBMC samples produced positive responses (>2.0 IU/ml). These results indicate that PBMCs in approxi-
We finally examined whether TNF-α production is also induced/enhanced in whole blood after IL-12 stimulation. Each of the whole blood samples stimulated with 1000 pg/ml rIL-12 was assayed for IFN-γ production and TNF-α concentration (Fig. 10). Patterns of IFN-γ production similar to those in Figs. 2 and 5 were observed in healthy individuals as well as in each PS group of cancer patients. Whereas IFN-γ concentration before stimulation was almost at the zero level in all samples, significant to considerable levels of TNF-α were detected in unstimulated blood irrespective of PS grades. After IL-12 stimulation, most of the healthy individuals and PS 0 patients produced enhanced levels of TNF-α. In contrast, only marginal or almost no enhancement of TNF-α production was observed, especially in PS 3 + 4 patients, which correlated with the failure of IL-12 to induce IFN-γ production. Thus, these results indicate that TNF-α production is enhanced in whole blood after IL-12 stimulation in association with the induction of IFN-γ production.

**DISCUSSION**

The results obtained in the present study show that compared to healthy individuals, cancer patients exhibit a substantial difference in IL-12 responsiveness as measured by IFN-γ production in whole blood after stimulation with IL-12. Namely, whole blood samples from all healthy subjects produced positive responses, although there was a considerable variation. In contrast, approximately half of the samples from cancer patients showed levels of IFN-γ production comparable to those for healthy samples, whereas the rest of the samples exhibited almost-null responses. The reduced IL-12 responsiveness did not necessarily correlate with the stages of the cancers, the number of lymphocytes contained in blood samples, or the tumor types; rather, it correlated with the PS of the patients.
The antitumor efficacy of IL-12 has been well investigated in various murine models, which revealed that the systemic administration of rIL-12 results in the complete regression or substantial inhibition of s.c. growing tumors (10-13). Although IFN-γ production is not sufficient to induce antitumor effects (18), IFN-γ production by T cells and NK cells is central to the scenario of IL-12-induced antitumor cascade reaction. Thus, assessing the capacity of PBMCs in cancer patients to produce IFN-γ in response to IL-12 would be the first screening test to determine whether the IL-12 effects can be expected.

The results obtained in our whole blood culture system demonstrate that IL-12 responsiveness, as measured by IFN-γ production after IL-12 stimulation in cancer patients, does not necessarily correlate with cancer stages but rather closely correlates with the PS of patients. The most remarkable feature is that in contrast to healthy subjects, some of the cancer patients produce null responses, and the incidence of null responders increases with the grade of PS. Differential IL-12 responsiveness was also expressed by TNF-α production in whole blood. Because TNF-α is produced mainly by macrophages depending on stimulation with T cell-derived IFN-γ (23-25), the enhancement of TNF-α production observed here seems to be secondary to IFN-γ production. Consistent with this, an almost complete correlation was observed between the enhanced production of TNF-α and the PS of the patients.

The concept of PS was first described as a criterion for evaluating the effects of chemotherapeutic drugs (26). Instead of focusing on the state of the tumor, PS takes into account any detrimental effects of the tumor on the host and may thereby represent the overall condition of the patient. PS has been considered to be a useful prognostic factor because of its correlation with survival (27-29). Nevertheless, the entity of PS remains to be clarified. This study suggests that PS may be reflected in the capacity of the patient to express immune responsiveness as exemplified here by IL-12-stimulated IFN-γ/TNF-α production.

IL-12 responsiveness was not determined by the number of PBMCs contained in whole blood. Namely, the mean value of lymphocyte numbers was slightly lower in the PS 1 + 2 and PS 3 + 4 groups than it was in a healthy control group, but the difference was not significant. Because the major responders to IL-12 are T cells and NK cells (1-3, 8), we analyzed the lymphocyte population from several cancer patients, especially from PS 3 + 4 patients. We found that there was no substantial difference in the percentages of CD56+ NK cells and CD3+CD56- T cells [CD56+, 18.6 ± 1.5 (healthy individuals) and 20.8 ± 3.5% (PS 3 + 4 patients); CD3+CD56-, 63.9 ± 1.8 (healthy individuals) and 52.6 ± 4.8% (PS 3 + 4 patients)]. Therefore, null IL-12 responsiveness is not ascribed to a lack of IL-12-responsive T cells/NK cells in the blood of patients.

The whole blood culture system used here was originally developed in our previous study (21). We showed that this system can assess the capacity of PBMCs contained in whole blood to produce cytokines, and that, in healthy individuals, there is a close correlation between cytokine production in whole blood and that in isolated PBMCs. The whole blood culture system has some advantages: (a) as little as 1 ml of venous blood permits us to examine the effect of IL-12 on IFN-γ production by the PBMCs; and (b) the actual event induced in subjects treated with IL-12 may be reflected in this system more correctly than in the assay in which isolated PBMCs are stimulated with IL-12 in the presence of FCS-positive culture medium instead of their own serum. However, it is also possible that IFN-γ production induced in the present system is modulated by various factors contained in the blood, although it may be the real in vivo event.

It has been shown that abnormally high levels of cytokines such as transforming growth factor β (30, 31), IL-6 (32-34), and IL-10 (35, 36) are detected in blood from tumor-bearing individuals. Each of these cytokines has been demonstrated to inhibit the production/secretion of a representative antitumor cytokine, IFN-γ, by T cells and NK cells (22, 32, 33). In this context, we found that when PBMCs from whole blood samples of some patients exhibiting null/marginal IL-12 responsiveness were isolated and stimulated with rIL-12 in culture medium, approximately half of these PBMC samples produced positive responses. These observations are consistent with the possibility that the cytokine-producing responses of T cells/NK cells in vivo or in the whole blood culture system could be modulated by serum factors and leukocytes coexisting in blood. However, we also found that PBMCs from samples still exhibited almost-null responses, suggesting the existence of an additional suppressive mechanism leading to the dysfunction of responding cells themselves. Whereas these two mechanisms were observed in PS 1 + 2 patients in this study, further analysis will be required to determine how the ratio of the two mechanisms changes with PS progression.
Our results illustrate that there is a substantial difference in IL-12-stimulated IFN-γ production by PBMCs among cancer patients, and such a difference correlates with the PS. IL-12-stimulated IFN-γ production is an initial essential process in the antitumor efficacy of IL-12. Thus, the present study could contribute not only to the selection of cancer patients who can exhibit the first step of the IL-12 effect but also to the development of approaches to analyze and correct the immunodysfunction occurring in patients with reduced IL-12 responsiveness.

ACKNOWLEDGMENTS

We thank the members of the Louis Pasteur Center for Medical Research and the Kyoto Red Cross Center for assistance and help with this work.

REFERENCES

Differential interleukin 12 responsiveness for interferon gamma production in advanced stages of cancer patients correlates with performance status.

K Uno, J Setoguchi, M Tanigawa, et al.


Updated version  Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/4/10/2425

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.