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H-2 Haplotype-Dependent Serum IL-12 Production in Tumor-bearing Mice Treated with Various Mycelial Extracts

AKIKUNI YAGITA, SYOJI MARUYAMA, SHINJI WAKASUGI and YASUSHI SUKEGAWA

Institute of Immunotherapy for Cancer, Kinki University Hospital, Osakasayama, Osaka 589-8511, Japan

Abstract. *IL-12 is considered to be one of the most important cytokines in anti-cancer therapy. We have demonstrated that substances derived from Basidiomycetes, such as active hexose-correlated compound (AHCC) and PSK induce the production of IL-12. In this study, the MHC dependency of IL-12 production induced by various mycelial extracts, PSK, AHCC and IL-X, was examined. During tumor-bearing, higher serum IL-12 levels were observed in H-2^a and H-2^b mice as compared to H-2^d mice. Concerning the effect of genetic background of mice on response to mycelial extracts, AIICC administration enhanced the serum IL-12 level in H-2^b mice but not in H-2^d mice, while PSK administration increased the serum IL-12 level in H-2^d mice but not in H-2^b mice. IL-X, components derived from the same Basidiomycetes, also enhanced the serum IL-12 level in H-2^b mice in the early stage of tumor like AHCC, and maintained serum IL-12 at a level higher than the normal value accompanying tumor growth, whereas AHCC did not restore the lowered serum IL-12 level accompanying tumor growth. These results showed that AHCC or IL-X is effective in a genetically Th1-dominant individual whereas PSK is effective in a genetically Th2-dominant individual or Th2-dominant status in advanced cancer patients. So we propose that the suitable combinations of various mycelial extracts may be effective methods of endogenous IL-12 induction for cancer patients of all stages, which is important as a cancer therapy that is relatively free from adverse reactions and which emphasizes the QOL in individual patients.*

Recent studies have elucidated that the balance of the activities of type1 helper T cells (Th1) and type 2 helper T cells (Th2) plays an important role in the regulation of immune response (1-3). As for the immune responses to tumors, Th1-induced activation of cellular immunity is considered to be the more important determinant. Interleukin-12 (IL-12) was originally reported as a substance that activates NK cell activity (4), but IL-12 also strongly augments IFNγ production (5-6), which induces Th1 activation. For this reason, IL-12 plays an extremely important role in the process of establishing of anti-tumor immune response (7) and is considered to be one of the most important cytokines in anti-cancer therapy. In animal studies, IL-12 administration has been reported to contribute to the establishment of anti-tumor immunity and the achievement of marked anti-tumor effect (8-11). In humans, administration of recombinant IL-12 has been attempted, in anticipation of an anti-tumor effect (12-13). However, due to strong adverse reactions, problems in clinical application of IL-12 have remained unsolved (14).

On the other hand, we have found that substances that induce IL-12 production exist in nature. Especially, we have demonstrated that substances derived from *Basidiomycetes,* such as active hexose correlated compound (AHCC) and PSK induce the production of IL-12.

PSK is a protein-bound polysaccharide extracted and purified from the cultured mycelia of *Coriolus versicolor Quél*, belonging to *Basidiomycetes.* In Japan, this substance is widely used as a drug that exhibits anti-tumor actions mediated by modification of the host immune competence (15-16). AHCC is an extract derived from the mycelia of *Basidiomycetes.* It has been shown to have an immunomodifying function and the active component is known to be α-1,4 bonded glucose oligomer. IL-X is a preparation composed from the mycelial extract of *Lentunus edodes* (shi-itake), the mycelium of *Schizophyllum commune* (suehirotake) and the mycelial extract of *Ganoderma lucidum* (reishi).

This paper reports on the capability of IL-12 production induced by the above substances which may differ depending on the major histocompatiblity complex (MHC)-type of the host and the tumor-bearing stage. In addition, based on the possibility that the capability of IL-12 production may clinically differ according to the patient's genetic background or pathological conditions, this report also discusses combined use of IL-12 inducers with different properties to increase the therapeutic effect against cancer.

Materials and Methods

Animals and tumors cells. Congenic strains of C57BL/10 mice; B10A, B10

Correspondence to: Akikuni Yagita, MD, Institute of Immunotherapy for Cancer, Kinki University Hospital, 377-2, Ohnohigashi, Osakasayama-city, Osaka 589-8511, Japan. Tel: +81(723)66-0221, Fax: +81(723)68-1132.

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Table I. *Serum IL-12 level in several mouse strains with different H-2 haplotypes.*

Strain	Haplotype			Serum IL-12 level (pg/mL)		Tumor
				Normal	Tumor bearer	
B10A	a	K^b	D^b	1779.3±210.5	3139.4±1130.5[a)]	L.L.C.
B10	b	K^k	D^d	1430.6±144.2	2126.1±306.5[b)]	"
B10D2	d	K^d	D^d	822.7±100.9	817.7±549.6	"
BALB/c	d	K^d	D^d	520.5±18.6	24 6.3±3 4.3[b)]	Colon 26

Sera were collected from non-tumor-bearing mice or tumor-bearing mice (day 10 after tumor inoculation), and serum IL- 12 levels were measured. Detailed methods are described in Materials and Methods.

a) normal *vs* tumor bearer $p < 0.01$
b) normal *vs* tumor bearer $p < 0.001$

and B10D2 were purchased from Japan SLC. BALB/c mice were purchased from Japan Charles River, Ltd. The mice were inoculated subcutaneously with tumor cells in the axilla region at 6 to 7 weeks of age. C57BL/10 mice were inoculated with 2×10^6 of Lewis lung carcinoma (LLC) cells, and BALB/c mice with 1×10^6 of colon 26 cells. Five to 6 mice were used in each group for each time of measurement.

Substances and methods of administration. AHCC was obtained from Amino-up Corp. (Sapporo, Japan) PSK (Krestin®) was obtained from Kureha Chemical Industry Co. Ltd. (Tokyo, Japan). IL-X was obtained from Noda Shokukin, (Noda, Japan), Taito (Tokyo, Japan), Royal Nutrition Japan, Ltd. (Tokyo, Japan). Krezyl was obtained from Sawai Pharmaceutical Co. Ltd. (Osaka, Japan). Each substance was administered orally at 1 g/kg everyday from one day after tumor inoculation.

Measurement of serum levels of IL-12. A mouse was laparotomized under ether anesthesia. Blood was collected from the abdominal large vein and the serum was separated. The serum level of IL-12 was measured by an IL-12 ELISA Kit (Genzyme Corp).

Results

(1) Difference in serum IL-12 production according to mouse H-2 haplotypes. Table I shows the serum levels of IL-12 in BALB/c mice and congenic C57BL/10 mice (B10A, B10 and B10D2) with different H-2 haplotypes, in non-tumor-bearing and tumor-bearing states (day 10 of tumor inoculation). Compared to B10D2 and BALB/c mice with H-2 haplotype "d", B10A mice with H-2 haplotype "a" and B10 mice with haplotype "b" showed higher serum IL-12 levels in both normal and tumor-bearing states. Furthermore, B10A and B10 mice had significantly higher IL-12 levels in tumor-bearing state than in normal state. On the other hand, no increase in serum IL-12 level was observed during tumor-bearing state in B10D2 mice, while the serum IL-12 level was significantly decreased during tumor-bearing state in BALB/c mice. The above results indicated that the IL-12 level produced during both tumor-bearing and non-tumor-bearing states differed depending on the H-2 haplotypes and that strains of mice with relatively high and relatively low IL-12 producing capability were present.

(2) Difference in response to IL-12 inducers according to mouse H-2 haplotypes. Table II shows the effects of oral administration of AHCC or PSK on the serum IL-12 levels during tumor-bearing state (day 10 of tumor inoculation) in B10 and B10D2 mice. In tumor-bearing B10 mice, PSK administration did not affect the serum IL-12 level, but AHCC administration significantly increased serum IL-12 level. In contrast, in B10D2 mice, AHCC administration did not induce marked changes in serum IL-12 level, but PSK administration significantly increased serum IL-12 level. The above results indicated that the response to inducers of IL-12 production also differed depending on the H-2 haplotype and that substances acting on high IL-12-producing strains and those acting on low IL-12 producing strains were present.

Table II. *Effects of AHCC or PSK administration on serum IL-12 level in mouse strains with different H-2 haplotypes.*

Strain	Serum IL-12 level (pg/mL)			
	Normal	Tumor bearer		
		Control	AHCC	PSK
B10	1430.6±144.2	2126.1±306.5[a)]	3232.4±282. 9[b)]	2034.64±366.2
B1OD2	822.7± 100.9	817.7 ±549.6	1003.6±477.6	1664.8±526.0[c)]

Mice were inoculated with Lewis lung carcinoma cells on day 0, and AHCC or PSK was orally administered every day from day 1. Sera were collected on day 10 after tumor inoculation, and serum IL- 12 levels were measured. Detailed methods are described in Materials and Methods.

a) normal *vs* control $p<0.001$
b) control *vs* AHCC $P<0.01$
c) control *vs* PSK $p<0.01$

(3) Tumor growth and IL-12 production. Using B10 mice, tumor weight and serum IL-12 levels were measured before tumor-bearing and on days 7, 10, 14 and 18 after tumor

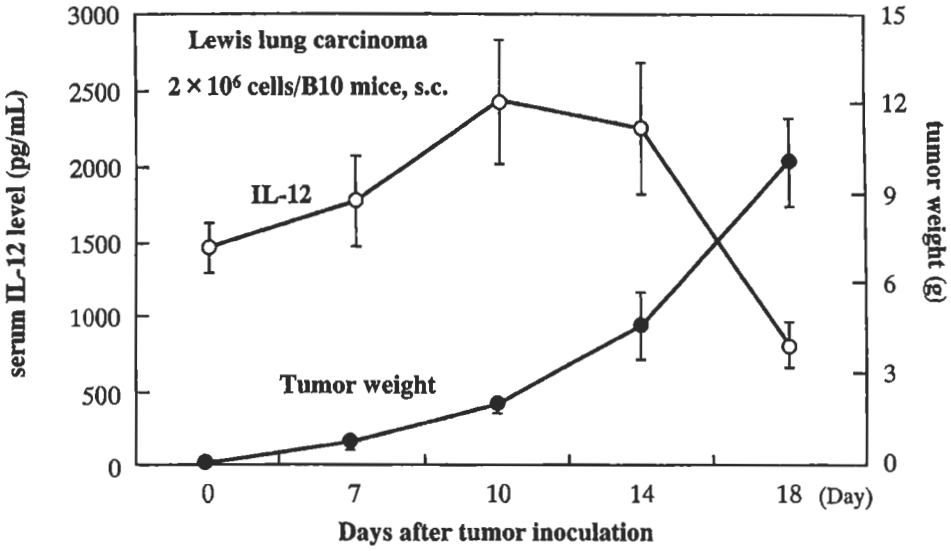

Figure 1. *Time-course of serum IL-12 production and tumor growth. B10 mice were inoculated with Lewis lung carcinoma cells and the tumor weights and serum IL-12 levels were measured on days 7, 10, 14 and 18 after tumor inoculation. Detailed methods are described in Materials and Methods.*

inoculation (Figure 1). Up to day 10 after tumor inoculation, the serum IL-12 level increased with increase in tumor weight. From day 14, rapid growth of the tumor was accompanied by an acute decline of serum IL-12 level. The above results indicated that in B10 mice, the level of IL-12 production is maintained during the initial stage of tumor growth but IL-12 production decreases markedly with the progression of tumor growth.

(4) Effects of AHCC and IL-X on the lowered IL-12 level accompanying tumor bearing. Tumor-bearing B10 mice were given oral administration of AHCC or IL-X, and the serum IL-12 levels were measured on days 7 and 14 of tumor inoculation. The results are shown in Figure 2. In the control group, increase in IL-12 level was observed on day 7 after inoculation, but decreased to below the normal level on day 14. In the AHCC group, although a tendency of increase in serum IL-12 level compared to the control group was observed on day 7 after tumor inoculation, the lowered serum IL-12 level accompanying cancer growth was not recovered to the normal level by AHCC administration on day 14. On the other hand, in the IL-X group, the serum Il-12 level tended to increase on day 7 of tumor-bearing and maintained a significantly higher level than the control group and a normal level on day 14.

(5) Effect of PSK and Krezyl on the lowered IL-12 level accompanying tumor bearing. PSK and Krezyl are both protein-bound polysaccharides derived from Basidiomycetes, wich are used clinically in Japan. The following experiment was conducted to examine if the IL-12-inducing capacities of these agents were different. Tumor-bearing B10 mice were orally administered PSK or Krezyl, and the serum IL-12 levels were measured on days 7, 10 and 16 of tumor inoculation. The results are shown in Table III. In the PSK group, the serum IL-12 level was maintained at the same or a slightly higher level as compared to the control group. On day 16 of tumor-bearing, a tendency of slight attenuation of the lowered serum IL-12 level was observed compared to the control group. On the other hand, in the Krezyl group, the serum IL-12 level was almost the same or slightly lower compared to the control group up to day 10 of tumor-bearing, becoming markedly decreased on day 16 of tumor bearing.

Discussion

The capacity of cytokine production has been reported to differ depending on the genetic background of the mice, especially on the H-2 haplotype. For example, we have reported that OK-432-stimulated TNF production is higher in B10A mice with H-2^a and B10 mice with H-2^b compared to B10D2 mice with H-2^d (17). In the present study, higher serum IL-12 levels during tumor-bearing were also observed in B10A mice with H-2^a and B10 mice with H-2^b compared to B10D2 mice and BALB/c mice both with H-2^d. Since TNF and IL-12 are Th1-type cytokines, we speculate that B10A mice with H-2^a and B10 mice with H-2^b exhibit a genetic dominance of Th1 cytokine production (these mice are called Th1 mice hereinafter), while B10D2 mice and BALB/c mice show a dominance in Th2 cytokine production (hereinafter called Th2 mice). Heinzel *et al.* (18) compared the effect of genetic background on Th1/Th2 cytokine balance in the mechanism of development of leishmaniasis in mice. They reported that the mRNA level of IFNγ (a Th1 cytokine) after leishmania infection was higher in C57BL/6 mice with H-2^b than in BALB/c mice with H-2^d, whereas the mRNA level of

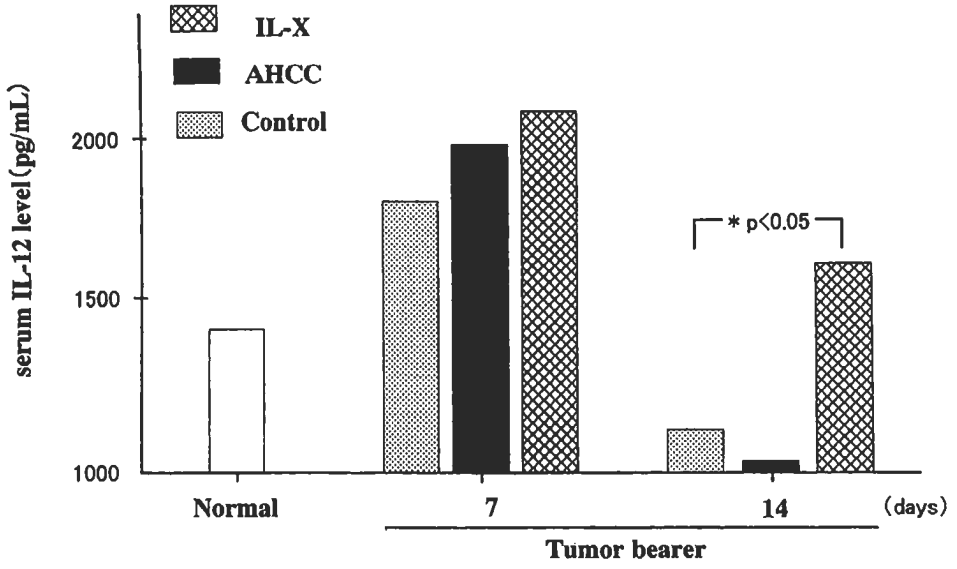

Figure 2. *Effect of AHCC or IL-X administration on the lowered IL-12 production accompanying tumor growth. B10 mice were inoculated with Lewis lung carcinoma cells on day 0, and AHCC or IL-X was orally administered everyday from day 1. Sera were collected on day 7 and 14 after tumor inoculation, and serum IL-12 levels were measured. Detailed methods are described in Materials and Methods.*

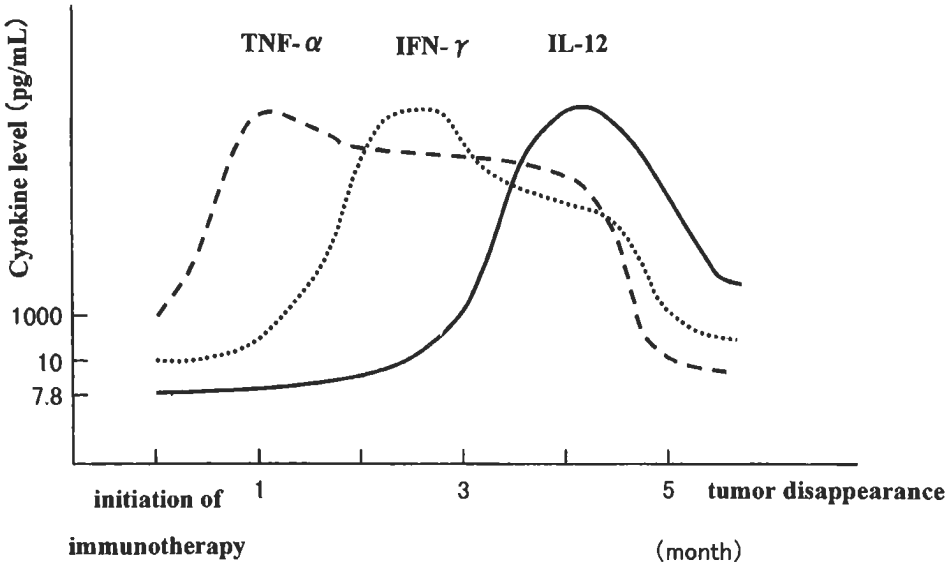

Figure 3. *Time course of Th1 cytokine production.*

IL-4 (a Th2 cytokine) was enhanced only in BALB/c mice with H-2^d.

Concerning the effect of genetic background of mice on response to mycelial extracts, AHCC administration enhanced the serum IL-12 level in B10 mice with H-2^b showing a dominance of Th1 cytokine, while PSK administration increased the serum IL-12 level in B10D2 mice with H-2^d exhibiting a dominance of Th2 cytokine. These results suggest that response to mycelial extracts is also controlled by the H-2 haplotype. These findings also suggest that PSK administration may result in shifting from a Th2 cytokine-dominant state to a Th1 cytokine-dominant state. Harada *et al.* (19) studied cytokine production in lymph node cells in DBA/2 mice inoculated into the subserosal space of

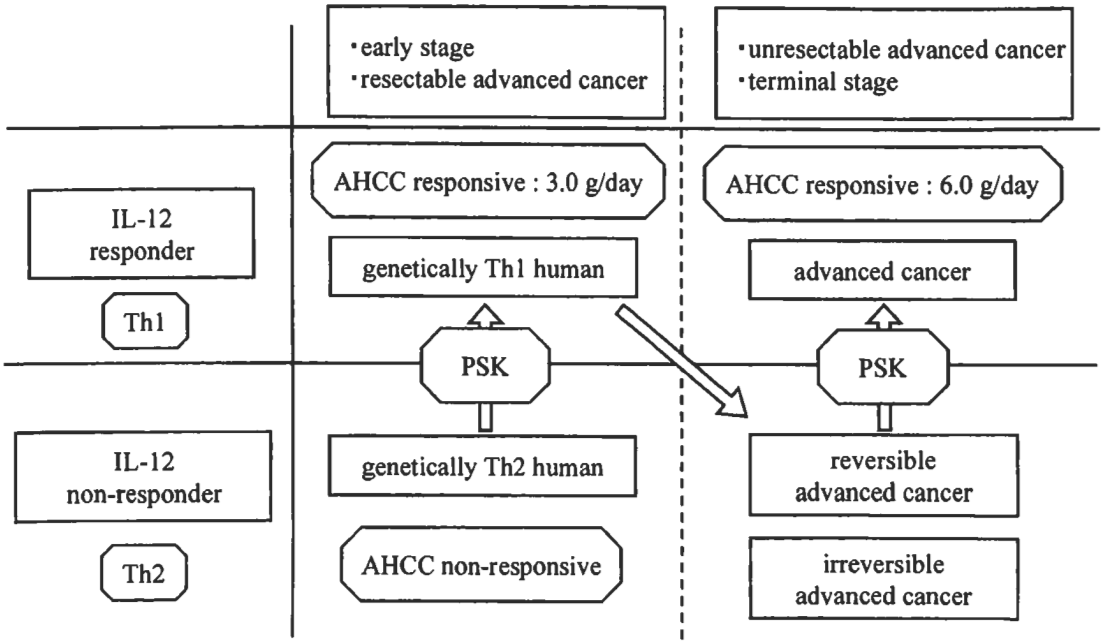

Figure 4. *Strategies for the induction of endogenous IL-12 in patients at various cancer stages. Explanations of the figure are given in Discussion.*

the cecum with human B7 (CD80) gene-transfected P815 cells. They reported that oral administration of PSK in these mice enhanced the production of IL-2 and IFNγ (Th1 cytokines) but suppressed IL-4 and TGF-β (Th2 cytokines).

Examination of the time courses of tumor growth and serum IL-12 levels showed (Figure 3) that although IL-12 was increased during the early stage of tumor inoculation in Th1 mice, IL-12 production declined markedly with tumor growth. This probably reflects a progression of immunosuppression accompanying tumor growth, which decreases the function of IL-12 production cells such as macrophages.

Our study also demonstrated that components derived from the same *Basidiomycetes* have different effects on the lowered serum IL-12 level accompanying tumor growth. IL-X not only induced IL-12 production in the early stage of tumor, but also attenuated the lowered serum IL-12 level accompanying tumor growth and maintained serum IL-12 at a level higher than the normal value. In contrast, although AHCC has an IL-12-inducing effect during the early stage of tumor, it does not restore the lowered serum IL-12 level accompanying tumor growth. Although the reason for this difference is unknown, differences in original material and production method may contribute to these results. The same difference can also be observed with PSK and Krezyl, both being protein-bound polysaccharides derived from *Coriolus versicolor*. While a tendency of attenuation of the lowered serum IL-12 level at the terminal stage of tumor-bearing was observed with PSK, the serum IL-12 level at the terminal stage of cancer remained markedly low with Krezyl

Table III. *Effects of PSK or Krezyl administration on serum IL-12 level in tumor-bearing B10 mice.*

Treatment	Serum IL-1 2 level (pg/mL)		
	Days after tumor inoculation		
	7	10	16
Normal	1707.8 ±162.3	1393.7±93.1	1549.7±200.3
Tumor bearer	1884.4±167.5	1929.2±647.5	1105.5±47 4.9
PSK(p.o.)	2067.6±207.5	2034.6±366.2	1299.0±464.0
Krezyl (p.o.)	1809.6±328.6	1709.2 ±643.9	303.7±883.1

Mice were inoculated with Lewis lung carcinoma cells on day 0, and PSK or Krezyl was orally administered every day from day 1. Sera were collected on day 7, 10 and 16 after tumor inoculation, and serum IL-12 levels were measured. Detailed methods are described in Materials and Methods.

administration. Differences in production method and purification method may also affect biological activity, but the exact cause remains unclear.

In the previous study, we found a difference in the time-course of Th1 cytokines-induction in cancer patients (20)(Figure 4). TNF-α is first induced after initiation of immunotherapy. When the TNF-α level reaches over 1,000 pg/ml, IFNγ production is consequently enhanced. When the IFNγ level increases over 10 pg/ml, then IL-12 is induced.

Although we did not measure TNF-α and IFNγ levels in the present study, PSK has been reported to induce TNF-α (21) and IFNγ (22). It is possible that this kind of time-lag in response may be associated with the induction of endogenous IL-12.

As a method of inducing endogenous IL-12 that has a high anti-tumor effect and no risk of adverse reactions, the present study examined various preparations of biological response modifiers. Our results showed that AHCC or IL-X are effective in a genetically Th1-dominant individual. Especially, we confirmed that IL-X was able to induce endogenous IL-12 even at the terminal stage of cancer-bearing. In a genetically Th2-dominant individual, however, AHCC alone failed to induce endogenous IL-12 and PSK was indispensable to induce a shift to Th1 dominance. Advanced cancer patients also become Th2-dominant (23), which leads to a strong immunosuppressive state. In this case, a possible strategy is first to achieve a Th1 shift with PSK and then add AHCC or IL-X to intensively induce endogenous IL-12.

Regarding the above considerations, systems of endogenous IL-12 induction have been proposed in Figure 4. In the stage of early cancers or resectable advanced cancers with residual immunocompetence, endogenous IL-12 may be induced by AHCC or IL-X alone in genetically Th1-dominant individuals, or by a combination of PSK and AHCC or IL-X in genetically Th2-dominant individuals. In the stage of unresectable advanced cancer or terminal cancer, even genetically Th1-dominant individuals become Th2-dominant. At such a stage, combined use of PSK and AHCC or IL-X is necessary in order to induce endogenous IL-12. Through such combinations, effective methods of endogenous IL-12 induction for cancer patients of all stages may be established, which is important as a cancer therapy that is relatively free from adverse reactions and which emphasizes the QOL in individual patients.

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