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# Impaired interferon-α production in whole-blood cultures from bladder cancer patients

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**Summary.** Interferon-α (IFN-α) production was investigated in whole-blood cultures of 66 bladder cancer patients and 65 control subjects. IFN synthesis was induced with Sendai virus, and IFN activity was assayed in FL cells challenged with vesicular stomatitis virus (VSV). The mean levels of the IFN-α produced were 5,724 ± 2,288 IU/ml in the control subjects and 4,800 ± 2,353 IU/ml in the bladder cancer patients. IFN-α production was significantly suppressed in the bladder cancer patients compared with that in the control subjects ($P < 0.05$). The impairment in IFN-α production correlated with the tumor grade, and it was shown that the tendency toward decreased IFN-α production was closely associated with the advancement of the tumor stage. Our results suggested that the decreased IFN-α production may contribute to the disordered immunoregulation in bladder cancer patients.

**Key words:** Bladder cancer – Impaired interferon-α production – Whole-blood culture – Cytopathic effect inhibition assay

It has been suggested that bladder cancer cells can induce both cell-mediated and humoral immune responses in immunocompetent hosts [2, 4, 6]. In addition, it has been found that bladder cancer patients have major impairment of their immune responses. Therefore, evaluation of immune defense mechanisms of the host with bladder cancer is of great importance in understanding the pathophysiology of the disease.

In 1957 Isaacs and Lindenmann [10] first reported interferon (IFN) to be a substance with antiviral activity. Since this original observation, IFN has been shown to have several biological activities, including antitumor [17] and immune modulation [5, 15]. Three types, IFN-α, IFN-β, and IFN-γ, have been identified and are classified on the basis of their antigenic and physicochemical properties. These IFNs act as an important potential constituent of the body's defense mechanism.

In animal models the neutralization of endogenously produced IFN by anti-IFN antibodies accelerates the progression of transplanted tumors [8]. In clinical investigations, patients with hematological malignancies were reported to have reduced the IFN-α production of peripheral leukocytes [9, 12, 14]. Recently, Skurkovich et al. [19] reported that IFN-α plays an important role in the initiation of an immune response. The disturbances in IFN-α synthesis can exert negative immunoregulatory effects on the organism. Therefore, it is interesting to examine the IFN-α production in bladder cancer patients at different stages of their tumors.

In general, the peripheral lymphocytes, isolated by Ficoll-Hypaque gradient centrifugation, are used for the routine laboratory determination of IFN-α production. However, this method is laborious and time-consuming. We employed a whole-blood method [11] to examine the IFN-α production. The advantages of this method are: (1) it is more simple than the leukocyte isolation method; (2) the IFN titers found in the whole-blood method are higher than those of the leukocyte-isolation method; (3) the whole-blood method may reflect the in vivo situation more strongly than the leukocyte-isolation method does.

In the present study, the IFN-α production was examined by the whole-blood method in bladder cancer patients in comparison with control subjects.

## Materials and methods

### *Patients*

A total of 66 patients with histologically verified bladder cancer were included in the study. The mean age of the patients was 68 ± 11 years (range 39 to 87), and the male-to-female ratio was 3.4:1. The patients did not receive any radiotherapy or chemotherapy for over 18 months before the IFN assay. Pathological diagnosis revealed transitional cell carcinoma in all cases. The histological grading (G category) and staging (T category) of the bladder cancers were done in accordance with the tumor, nodes and metastasis classification of the International Union Against Cancer. The distributions are shown in Table 1. As control subjects, we used 20 healthy donors and

**Table 1.** Distribution of the grades and stages in 66 bladder cancer patients

| Grade \ Stage | pTa | pT1 | pT2 | pT3 | pT4 | Metastasis | Total |
|---|---|---|---|---|---|---|---|
| I | 15 | 2 | 0 | 0 | 0 | 0 | 17 |
| II | 10 | 8 | 6 | 0 | 0 | 5 | 29 |
| III | 1 | 3 | 3 | 7 | 3 | 3 | 20 |
| Total | 26 | 13 | 9 | 7 | 3 | 8 | 66 |

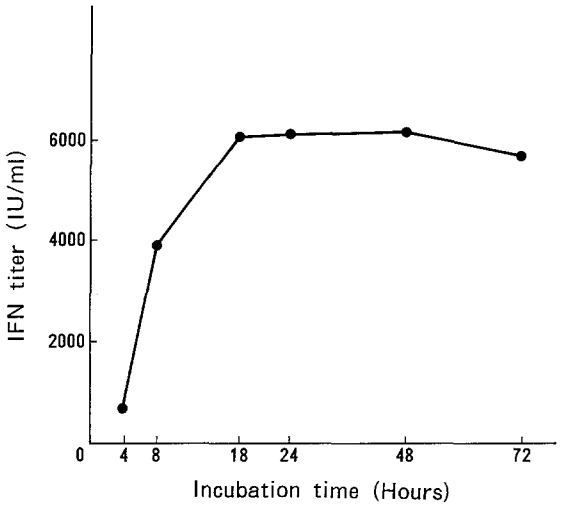


**Fig. 1.** Kinetics of IFN production in the whole-blood cultures after induction with Sendai virus. The data in this figure are from one of four representative experiments

45 patients with benign urological disease, the mean age being 64 ± 13 (range 38 to 81), and the male-to-female ration 3.1:1.

### *In vitro production of IFN by whole-blood cultures*

Two milliliters of blood was collected in heparinized tubes. Sendai virus was added at a final concentration of 500 HA units/ml within 5 h after blood drawing. The blood-virus mixture was incubated at 37°C for 20 h in a humidified incubator in 5% $CO_2$ with 95% air. The culture supernatants were harvested by centrifugations and stored at −70°C until assay for IFN was done.

### *IFN-α characterization*

*Neutralization of IFN by anti-IFN sera.* Neutralization was performed by incubating the IFN preparation for 1 h at 37°C with an equal volume of the following antisera: polyclonal sheep anti-IFN-α (Japan Chemical Research, Japan), monoclonal antibody against anti-IFN-β (Toray Industries, Japan), and monoclonal antibody against IFN-γ (Green Cross Corp., Japan). After neutralization, the residual IFN activity was assayed.

*Acid stability of the IFN.* The IFN preparations were dialyzed against 0.1 M glycin-HCl buffer (pH 2) for 24 h and then against phosphate-buffered saline (pH 7.2) for 24 h. The residual IFN activity was assayed.

### *Cytopathic effect (CPE) inhibition assay for IFN-α production*

The antiviral activity of the culture supernatants was determined by the dye-uptake method using vesicular stomatitis virus (VSV) and human amnion FL cells. The culture supernatants (50 µl) were serially diluted twice in the wells of flat-bottomed 96-well microtiter plates (Corning, New York). Next, 50 µl of FL cells suspended ($1 \times 10^6$/ml) in Eagle's MEM containing 5% fetal calf serum (Flow Laboratories Inc., USA) was added to all wells. After incubation at 37°C for 24 h, the supernatants were removed, and the cells were challenged with VSV in 50 µl at a concentration of ten tissue culture infectious doses ($TCID_{50}$) per ml, and incubated another 30 h. The cells were stained with 1% neutral red (pH 6.7) for 1 h and then carefully washed twice with phosphate-buffered saline and dried at room temperature. For quantitative assay, the dye was extracted by adding 0.2 ml of dye extract solution (0.1 mol/l sodium phosphate:ethanol = 1:1), and the absorbance of the extracted dye was measured in a spectrophotometer (Titertek Multiskan, Helsinki, Finland) at 546 nm. The reciprocal of IFN concentration which reduced the absorbance at 546 nm by 50% was expressed as laboratory IFN units, and these values were calibrated against the MRC reference IFN standard preparation (code 69/19) and expressed as international units (IU). Each assay was performed in triplicate.

### *Analysis of total and differential Leukocyte counts*

In 46 bladder cancer patients and 38 control subjects, blood was drawn to count the total leukocytes at the same time of the IFN-α production, by using an automatic counter. Absolute and relative lymphocyte and monocyte counts were calculated from the results of the differential leukocytes after the examination of Wright-stained smears.

### *Statistical analysis*

Analysis of variance and paired t tests were used to analyze the data. When the $P$ value was less than 0.05, the difference was judged to be significant.

## Results

### *Production of IFN-α by Sendai virus in whole-blood cultures*

The kinetics of IFN production were examined in four healthy individuals. Two milliliters of whole blood was exposed to Sendai virus (500 HA units/ml) and cultured at 37°C. Significant IFN activity was detected in the supernatant of the culture fluid 4 h after incubation. The IFN

**Table 2.** Type of IFN induced by the Sendai virus in whole blood cultures[a]

| Experiments | Residual IFN titer after treatment with (IU/ml) | | | | |
|---|---|---|---|---|---|
| | None | Anti-IFN-α | Anti-IFN-β | Anti-IFN-γ | pH 2 |
| 1 | 6,110 | 18 | 6,410 | 6,330 | 5,980 |
| 2 | 5,940 | 9 | 6,230 | 6,430 | 6,010 |

[a] Whole peripheral blood from two healthy individuals was incubated with Sendai virus at 37°C for 20 h. After incubation, the culture supernatants were harvested by centrifugation. Then characterization of IFN in the culture supernatants was determined as described in Materials and methods

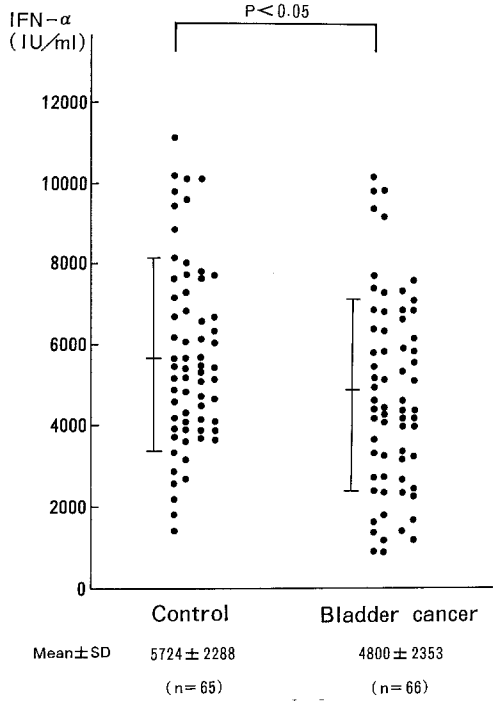


**Fig. 2.** IFN-α production in whole-blood cultures from bladder cancer patients and control subjects. IFN synthesis was induced with Sendai virus, and IFN activity was determined by the cytopathic effect inhibition assay as described in the text. The results of two groups are presented. The *horizontal bar* represents the mean ± SD

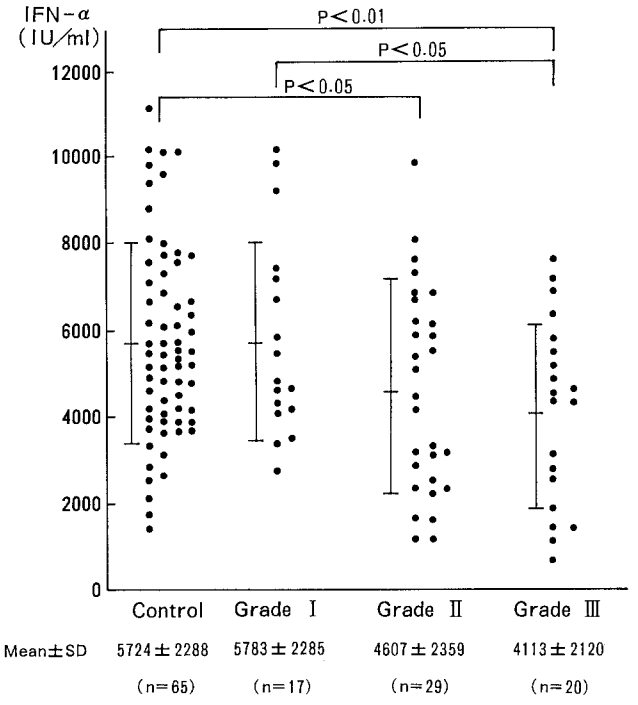


**Fig. 3.** IFN-α production in the different grades of bladder cancer patients. The methods of IFN production and assay are described in Fig. 2. The *horizontal bar* represents the mean ± SD

titer reached its maximum in 18–24 h and retained its level for at least 72 h (Fig. 1). Thus, in the subsequent experiments, IFN titers were measured in the supernatant harvested after a 20-h incubation period.

*Characteristics of IFN-α*

The IFN activity was completely neutralized by anti-IFN-α, but not by anti-IFN-β or anti-IFN-γ sera. It was stable with pH 2 treatment (Table 2). Thus, it was concluded that the IFN induced by the Sendai virus in whole-blood cultures was IFN-α.

*IFN-α production in controls and bladder cancer patients*

The mean activity of IFN-α produced in 65 control cases was 5,724 ± 2,288 IU/ml, while that of 66 bladder cancer patients was 4,800 ± 2,353 IU/ml. There was a significant difference in IFN-α production between the control subjects and bladder cancer patients ($P < 0.05$) (Fig. 2).

*IFN-α production in the different grades of bladder cancer*

IFN-α production was examined in the different grades of bladder cancer patients and control subjects (Fig. 3). There was no difference in the IFN-α production between the grade I and the control subjects. The IFN-α activity produced in grade II was significantly lower thant that in the control subjects ($P < 0.05$), and the IFN-α production

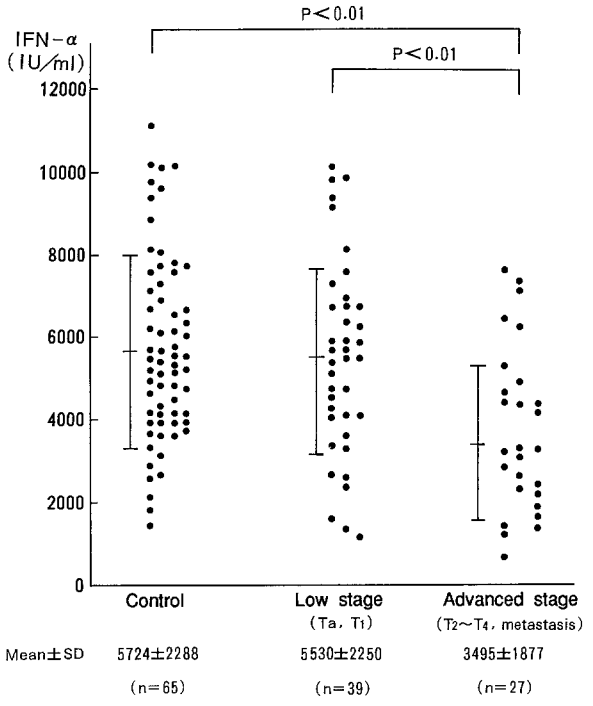


**Fig. 4.** IFN-α production in the low and advanced stages of bladder cancer patients. The methods of IFN production and assay are described in Fig. 2. The *horizontal bar* represents the mean ± SD

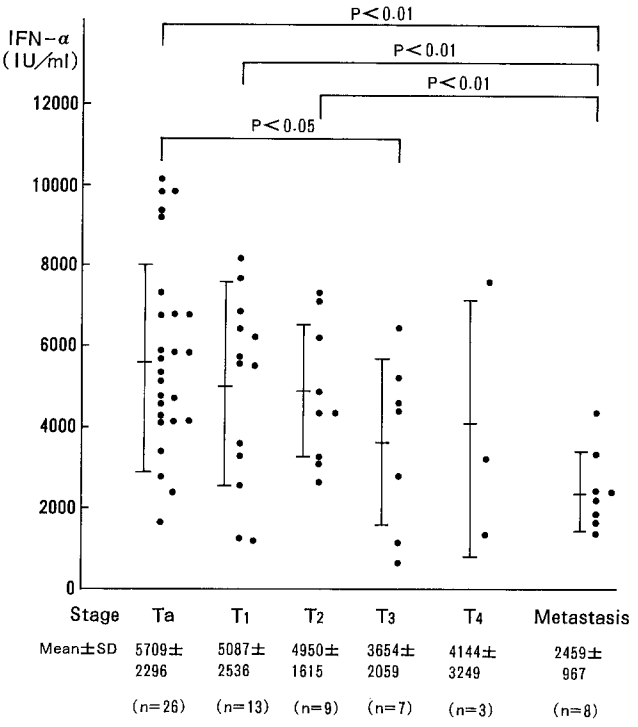


**Fig. 5.** IFN-α production in the different stages of bladder cancer patients. The methods of IFN production and assay are described in Fig. 2. The *horizontal bar* represents the mean ± SD

**Table 3.** Peripheral leukocytes, lymphocytes, and monocytes in the different stages of bladder cancer patients and control subjects

| | No. samples | Total leukocyte ($\times 10^3/mm^3$) | Lymphocyte ($\times 10^3/mm^3$) | Monocyte ($\times 10^3/mm^3$) |
|---|---|---|---|---|
| Bladder cancer | 46 | 5.4 ± 1.3 | 1.7 ± 0.6 | 0.23 ± 0.14 |
| Stage Ta | 17 | 5.3 ± 1.1 | 1.8 ± 0.6 | 0.19 ± 0.15 |
| Stage T1 | 10 | 4.9 ± 1.4 | 1.4 ± 0.2 | 0.37 ± 0.16 |
| Stage T2 | 6 | 5.4 ± 0.7 | 1.7 ± 1.0 | 0.32 ± 0.16 |
| Stage T3 | 5 | 6.1 ± 2.3 | 1.8 ± 0.5 | 0.21 ± 0.13 |
| Stage T4 | 2 | 4.9 ± 0.4 | 1.8 ± 0.7 | 0.30 ± 0.09 |
| Metastasis | 6 | 5.6 ± 1.5 | 1.4 ± 0.5 | 0.22 ± 0.06 |
| Control subjects | 38 | 5.7 ± 1.4 | 1.9 ± 1.0 | 0.30 ± 0.19 |

[a] Values are expressed as mean ± SD

**Table 4.** Peripheral leucocytes, lymphocytes, and monocytes in the different grades of bladder cancer patients and control subjects[a]

| | No. samples | Total leukocyte ($\times 10^3/mm^3$) | Lymphocyte ($\times 10^3/mm^3$) | Monocyte ($\times 10^3/mm^3$) |
|---|---|---|---|---|
| Bladder cancer | 46 | 5.4 ± 1.3 | 1.7 ± 0.6 | 0.23 ± 0.14 |
| Grade I | 11 | 5.6 ± 1.0 | 1.8 ± 0.8 | 0.27 ± 0.18 |
| Grade II | 22 | 5.1 ± 1.0 | 1.7 ± 0.6 | 0.21 ± 0.14 |
| Grade III | 13 | 5.7 ± 1.7 | 1.5 ± 0.5 | 0.25 ± 0.12 |
| Control subjects | 38 | 5.7 ± 1.4 | 1.9 ± 1.0 | 0.30 ± 0.19 |

[a] Values are expressed as mean ± SD

in grade III was significantly lower than that in the control subjects and grade I ($P < 0.01$, $P < 0.05$, respectively). The IFN-α production in grade III seemed to be lower than that in grade II, but the difference was not statistically significant.

*IFN-α production in the different stages of bladder cancer*

IFN-α production in the different stages of bladder cancer patients is presented in Figs. 4 and 5. There was no significant difference between the low stage (Ta, T1) and the control subjects, but the IFN-α production in the advanced stage (T2–4, metastasis) was lower than that in the low stage and in the control subjects ($P < 0.01$). When stage subgroups were studied separately, the IFN-α production tended to decrease according to the advancement of the tumor stage. The IFN-α production in stage T3 was significantly lower than that in stage Ta ($P < 0.05$). The metastatic group showed significantly low IFN production, compared to the control subjects and stage Ta, T1 and T2 patients ($P < 0.01$). However, the number of patients with stage T4 was small, and there was no significant difference from the other stages.

*Total leukocyte, lymphocyte and monocyte counts*

In order to determine whether the impairment of IFN production was due to the depletion of leukocytes, lymphocytes or monocytes, we analyzed the peripheral blood of 38 control subjects and 46 bladder cancer patients. Total leukocyte, lymphocyte and monocyte counts showed no large variations between bladder cancer patients and control subjects, and there was no significant difference among the different stages and grades in the cancer group (Tables 3 and 4).

## Discussion

The demonstration of immune reactivity in neoplastic diseases has led to considerable clinical significance. Recently, the determination of IFN production in human peripheral leukocytes by using a virus as an inducer has become one of the standard methods for clinical studies [7]. Lee and Hadhazy have reported that in vitro IFN production by peripheral leukocytes is markedly depressed in patients with leukemia [9, 12]. They suggest that this probably reflects a general impairment of lymphocyte function. The leukocytes of patients with some diseases, including Hodgkin's disease, systemic lupus erythematosus, and immunodeficiency associated with uremia have been shown to produce abnormally low levels of IFN-α in vitro in response to appropriate inducers [13, 14, 18].

The IFN production by the blood cells of patients with a solid tumor has received little attention. The present study is the first to provide information on the IFN-α production in bladder cancer patients. Our results show that the IFN-α production was decreased in the bladder cancer patients compared with that in the control subjects. We observed nearly normal IFN-α production in the low stage (stage Ta, T1) and in the low grade (grade I), but decreased production in the advanced stage (stage T2–T4, metastasis) and in the high grade (grade III). We found that decreased IFN-α production ws correlated with the tumor grade, and the tendency toward decreased IFN-α production was closely associated with the advancement of the tumor stage. The results suggested that tumor aggressiveness appeared to reduce the IFN-α production. However, the present data are not sufficient to answer the question as to whether IFN deficiency is due to a primary defect, to a secondary one caused by the malignant process, or to both.

The finding of decreased IFN-α production in bladder cancer patients and the inhibitory effect of IFN-α on in vitro growth of human bladder cancer cells [1] might suggest that exogenous IFN-α could be beneficial for bladder cancer patients. However, the in vivo application of IFN-α was not always consistent with the in vitro findings. Systemic administration of IFN-α does not show a significant effect in bladder cancer patients [21].

We examined IFN production in whole-blood cultures exposed to Sendai virus, which is most commonly used because of its high efficiency as an inducer [3, 20]. There are some reports concerning IFN production by using Sendai virus in human peripheral blood mononuclear cells. Saksela et al. [16] have reported that the major, and perhaps the only, producing cells were the monocytes when human leukocyte suspensions were infected with Sendai virus. Yamaguchi et al. [22], however, suggest that the producing cells an lymphocytes, especially B lymphocytes. The conflicting results may be due to the methods applied. In the present study, there were no large variations in the peripheral lymphocyte and monocyte counts between bladder cancer patients and the control subjects. There was no correlation with the grade and stage of the tumor. Thus, it is suggested that the low IFN production in bladder cancer patients did not result from a decreased number of lymphocytes or monocytes. Although the mechanism of impaired IFN-α production in bladder cancer patients is not yet clarified, two possible explanations could be: (1) that circulating IFN inactivators are present in the serum of the patients; (2) that the function of IFN-α producing cells is impaired.

It is important to determine whether the restoration of decreased IFN-α production correlates with a clinical cure. This will enable us to gain a better understanding of the IFN system in bladder cancer patients and might lead towards a more efficient therapeutic use of IFN.

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## References

1. Borden EC, Groveman DS, Nasu T, Reznikoff C, Bryan GT (1984) Antiproliferative activities of interferons against human bladder carcinoma cell lines in vitro. J Urol 132:800

2. Bubenik J, Perlmann P, Helmstein K, Moberger G (1970) Cellular and humoral immune responses to human urinary bladder carcinomas. Int J Cancer 5:310
3. Cantell K, Hirvonen S (1981) Preparation and assay of Sendai virus. Methods Enzymol 78:299
4. Catalona WJ, Chretien PB, Trahan EE (1974) Abnormalities of cell-mediated immunocompetence in genitourinary cancer. J Urol 111:229
5. Djeu JY, Heinbaugh JA, Holden HT, Herberman RB (1979) Augmentation of mouse natural killer cell activity by interferon and interferon inducers. J Immunol 122:175
6. Elhilali MM, Nayak SK (1975) Immunologic evaluation of human bladder cancer: in vitro studies. Cancer 35:419
7. Gresser I (1961) Production of interferon by suspensions of human leucocytes. Proc Soc Exp Biol Med 108:799
8. Gresser I, Belardelli F, Maury C, Maunoury MT, Tovey MG (1983) Injection of mice with antibody to interferon enhances the growth of transplantable murine tumors. J Exp Med 158:2095
9. Hadhazy G, Gergely L, Toth FD, Szegedi G (1967) Comparative study on the interferon production by the leucocytes of healthy and leukaemic subjects. Acta Microbiol Acad Sci Hung 14:391
10. Isaacs A, Lindenmann J (1957) Virus interference. I. The interferon. Proc R Soc Lond 147:258
11. Kita M, Kishida T (1987) An improved method for measuring human interferon-alpha producing capacity. Bull Inst Pasteur Kyoto 1:1 (in Japanese)
12. Lee SHS, Ozere RL, van Rooyen CE (1966) Interferon production by human leukocytes in vitro. Reduced level in lymphatic leukemia. Proc Soc Exp Biol Med 122:32
13. Neighbour PA, Grazel AI (1981) Interferon production in vitro by leucocytes from patients with systemic lupus erythematosus and rheumatoid arthritis. Clin Exp Immunol 45:576
14. Rassiga-Pidot AL, McIntyre OR (1974) In vitro leukocyte interferon production in patients with Hodgkin's disease. Cancer Res 34:2995
15. Ratliff TL, McCool RE, Catalona WJ (1981) Interferon induction and augmentation of natural-killer activity by Staphylcoccus Protein A. Cell Immunol 57:1
16. Saksela E, Virtanen I, Hovi T, Secher DS, Cantell K (1984) Monocyte is the main producer of human leukocyte alpha interferons following Sendai virus induction. Prog Med Virol 30:78
17. Salvin SB, Youngner JS, Nishio J, Neta R (1975) Brief communication: tumor suppression by a lymphokine released into the circulation of mice with delayed hypersensitivity. J Natl Cancer Inst 55:1233
18. Sanders CV, Luby JP, Sanford JP, Hull AR (1971) Suppression of interferon response in lymphocytes from patients with uremia. J Lab Clin Med 77:768
19. Skurkovich S, Skurkovich B, Bellanti JA (1987) A unifying model of the immunoregulatory role of the interferon system: can interferon produce disease in humans? Clin Immunol Immunopathol 43:362
20. Strander H, Cantell K (1966) Production of interferon by human leukocytes in vitro. Ann Med Exp Fenn 44:265
21. Taguchi T, Takakura K, Kimura K, Ohta K, Izuo M, Koyama Y, Niijima T, Ikeda S, Akaboshi Y (1983) Clinical studies of recombinant leukocyte α interferon (r-IFN-α, Ro22-8181). Proceedings of the International Symposium on Interferons. Japan Convention Services, Kyoto, p 173
22. Yamaguchi T, Handa K, Shimizu Y, Abo T, Kumagai K (1977) Target cells for interferon production in human leukocytes stimulated by Sendai virus. J Immunol 118:1931

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