# Liver Targeting of Interferon Through Pullulan Conjugation

**Keli Xi,[1,2] Yasuhiko Tabata,[1] Kazuko Uno,[3] Miwa Yoshimoto,[3] Tsunataro Kishida,[3] Yoshihiro Sokawa,[4] and Yoshito Ikada[3,5]**

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***Purpose.*** The purpose of this study was to actively target interferon (IFN) to the liver through its chemical conjugation with pullulan, a water-soluble polysaccharide with a high affinity for the liver.

***Methods.*** Chemical conjugation of IFN with pullulan was achieved by a cyanuric chloride method. Following intravenous injection of the conjugates to mice, their body distribution and the activity of an IFN-induced enzyme, 2′,5′-oligoadenylate (2-5A) synthetase in the liver and other organs, were evaluated.

***Results.*** The cyanuric chloride method enabled us to prepare an IFN-pullulan conjugate that retained approximately 7–9 % of the biological activity of IFN. Pullulan conjugation enhanced the liver accumulation of IFN and the retention period with the results being reproducible. When injected intravenously to mice, the IFN-pullulan conjugate enhanced the activity of 2-5A synthetase in the liver. The activity could be induced at IFN doses much lower than those of free IFN injection. In addition, the liver 2-5A synthetase induced by conjugate injection was retained for 3 days, whereas it was lost within the first day for the free IFN-injected mice.

***Conclusions.*** IFN-pullulan conjugation was promising for IFN targeting to the liver with efficient exertion of its antiviral activity therein.

**KEY WORDS:** interferon; pullulan conjugation; liver targeting; 2-5A synthetase.

## Introduction

Several clinical studies have demonstrated that interferon (IFN) therapy is effective in controlling the disease activity in patients with chronic infection of hepatitis C virus (1). However, several side effects have often induced clinical problems (e.g. flu-like symptoms, leukopenia, and psychoneurosis) during IFN therapy. One method to minimize the side effects is to reduce the injection dose of IFN by targeting to its site of action, the liver.

In the liver targeting approach, two different strategies can be distinguished: passive and active targeting. In the case of passive targeting, a carrier-associated drug is delivered by normal physiological processes to the liver. For example, the inevitable capture of intravenously injected particulate carriers by the reticuloendothelial system in the liver result in liver targeting of drugs (2). The vasculature of the liver characterized by free extravasation for most water-soluble substances circulating in the blood and by the negatively charged cell surface promotes the passive targeting of a positively charged water-soluble polymer to the liver (3). Active targeting, on the other hand, is based on the biospecific recognition mechanism like ligand-receptor interaction. A number of trials on the active targeting to the liver have been evaluated by taking advantage of asialoglycoprotein (galactose) receptors on the liver cells (4,5).

The present study aims at active targeting of IFN to the liver through its chemical conjugation with pullulan which is a water-soluble polysaccharide with a high affinity for the liver (6). After intravenous (iv) injection of the IFN-pullulan conjugate to mice, its accumulation in the liver and the activity of liver 2′,5′-oligoadenylate (2-5A) synthetase, an IFN-induced enzyme, were investigated.

## MATERIALS AND METHODS

### Mice

Specific pathogen-free inbred female BALB/cCrSlc mice aged 4–6 months were obtained from Shizuoka Animal Facility Center, Shizuoka, Japan.

### IFN

Recombinant human IFN αA/D ($1 \times 10^8$ IU/mg protein) was kindly supplied by Nippon Roche Research Center, Kamakura, Japan. This IFN is the product of a hybrid DNA of A and D clones, and is effective against mouse cells as well as human cells. The endotoxin level in the original preparation was determined by the Limulus test to be 1.6 ng/100μg IFN ($1 \times 10^7$ IU) or less.

### Reagents

Pullulan with a weight-average molecular weight of 200,000 was purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan and $Na^{125}I$ aqueous solution in $10^{-5}$ M NaOH (740 MBq/ml) from NEN Research Products, Boston, MA. Phosphate-buffered saline solution (PBS, pH 7.4) and HEPES were purchased from Nissui Seiyaku Co. Ltd., Tokyo, Japan. Other reagents were purchased from Nacalai Tesque, Inc., Kyoto, Japan and used without further purification.

### Conjugation of IFN to Pullulan

Conjugation of IFN to pullulan was carried out using cyanuric chloride (CA) (7). CA solution of various concentrations in dimethyl formamide (1 ml) was added to 3 ml of aqueous solution containing 50 mg of pullulan at pH 8.5, followed by stirring for different periods of time at 4°C to introduce CA residues into the hydroxyl groups of pullulan. The resulting mixture was filtrated through a PD-10 column (Pharmacia Biotech AB, Uppsala, Sweden) to separate the nonreacted CA from the cyanurated pullulan. Then, 0.3 ml of IFN solution ($1.68 \times 10^{-9}$ mole) in 0.05M phosphate buffer solution containing 0.05 M NaCl (PB, pH 7.0) was added to various concentrations of cyanurated pullulan with different percentages of CA introduction. After conjugation at 4°C for 12 hr, the reaction mixture

[1] Research Center for Biomedical Engineering, Kyoto University, 53 Kawahara-cho Shogoin, Sakyo-ku, Kyoto 606, Japan.
[2] Present address: Chemistry Department, Yunnan University, Kunming 650031, China.
[3] Institut Pastuer de Kyoto, 103-5 Monzen-cho, Tanaka, Sakyo-ku, Kyoto 606, Japan.
[4] Department of Biotechnology, Kyoto Institute of Technology, Gosyo-kaido-cho, Matsugasaki, Sakyo-ku, Kyoto 606, Japan.
[5] To whom correspondence should be addressed.

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was subjected to gel filtration on a Sephacryl S-200 column (0.5 × 36 cm) at a PB flow rate of 0.33 ml/min. The pullulan and IFN in each fraction were determined according to the sulfuric acid-anthrone method and the trinitrobenzene sulfonic acid method, respectively. The IFN activity was estimated from its antiviral activity (8). The amount of IFN was compared on the basis of the biological activity and indicated in terms of the antiviral activity detected in the IFN-pullulan conjugate. $^{125}$I-labeled IFN prepared (9) was similarly conjugated to pullulan.

### Body Distribution of IFN-Pullulan Conjugate

Free $^{125}$I-labeled IFN ($10^3$ IU) and 94.3 μg IFN-pullulan conjugate containing $10^3$ IU of $^{125}$I-labeled IFN were injected intravenously into BALB/c mice (5 mice/group). The mixture of 94.3 μg of IFN-free pullulan and $10^3$ IU of free $^{125}$I-labeled IFN was also injected as a control. At different time intervals, blood was collected by direct bleeding from the heart of anesthetized mice. Then, organs were removed, followed by twice rinsing with PBS. The radioactivity of the blood sample, each organ, and the carcass (the residual body parts) was counted in an autowell gamma counter (ARC-300, Aloka Co. Ltd., Tokyo, Japan). The total radioactivity of urine and feces was also measured for excreted samples.

### *In Vivo* Experiments

Mice were injected intravenously with $10^2$, $10^3$, $10^4$, $10^5$, or $10^6$ IU of free IFN and 94.3 μg of IFN-pullulan conjugate containing 10, $10^2$, or $10^3$ IU of IFN in 0.1 ml of PBS. As a control, 94.3 μg of IFN-free pullulan alone was injected. At different time intervals, the liver, lung, and spleen were removed from the mice, frozen immediately in liquid nitrogen and stored at −85°C until assay of their 2-5A synthetase level.

### Detection of 2-5A Synthetase

The 2-5A synthetase of the excised organ was detected using Western blotting and by the use of a rat monoclonal antibody to mouse 42-kD 2-5A synthetase which was prepared by Sokawa et al. (10). It is believed that the 30-kD protein sharing the same epitope with the 42-kD 2-5A synthetase is a breakdown product of the 42-kD protein, or a new member of the IFN-induced proteins in mouse cells.

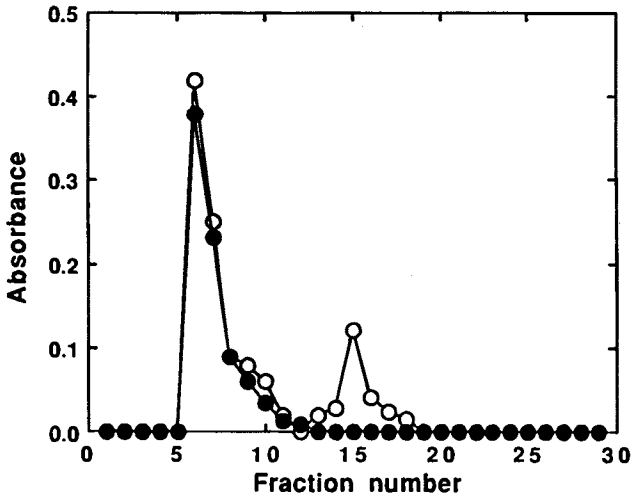


**Fig. 1.** Gel exclusion chromatography of IFN-pullulan conjugate. 1.68 × $10^{-9}$ mole (5.7 × $10^7$ IU) IFN was conjugated to 1.50 × $10^{-7}$ mole of cyanulated pullulan after CA introduction by 0.53 mole%. (○) the protein amount detected by the trinitrobenzene sulfonic acid method and (●) the pullulan amount detected by the sulfuric acid-anthrone method.

## RESULTS

### Conjugation of IFN to Pullulan

The molar percentage of CA introduced to the hydroxyl groups of pullulan depended on the molar ratio of CA/pullulan, the solution pH, and the reaction time (Xu K, et al, Unpublished data, December 1995). The percentage of CA introduced increased with increasing solution pH and reaction time up to 24 hr and thereafter leveled off, irrespective of the solution type.

From gel filtration chromatography, two elution peaks of protein were obtained in the fractions 6–10 and 13–18 (Figure 1). Clearly, the former peak is attributed to the IFN-pullulan conjugate because the peak position of pullulan was in good accord with that of the protein fraction of higher molecular size. When globular proteins with different molecular weights were used to calibrate the column, the latter peak was located near the molecular weight of IFN, indicating that it corresponded to free IFN.

The total amount of proteins in the conjugate ranged from 70.2 to 88.7% of the protein amount initially added (Table 1). Each value was similar to that of IFN incorporated in the

**Table I.** Conjugation of IFN to Cyanurated Pullulan

| IFN (mole) | Cyanurated Pullulan (mole) | Percent Cyanurated (mole %) | Amount of IFN Conjugated (mole) | Activity Remaining (%) | Pullulan Recovered (%) |
|---|---|---|---|---|---|
| 1.68 × $10^{-9}$ | 1.5 × $10^{-7}$ | 0.53 | 1.18 × $10^{-9}$ (70.2)[a] | 7.0 | 95 |
| 1.68 × $10^{-9}$ | 1.5 × $10^{-7}$ | 0.95 | 1.24 × $10^{-9}$ (73.8) | 7.5 | 94 |
| 1.68 × $10^{-9}$ | 1.5 × $10^{-7}$ | 1.5 | 1.26 × $10^{-9}$ (75.0) | 7.7 | 95 |
| 1.68 × $10^{-9}$ | 4.2 × $10^{-7}$ | 1.5 | 1.42 × $10^{-9}$ (84.5) | 8.9 | 95 |
| 1.68 × $10^{-9}$ | 5.2 × $10^{-7}$ | 1.5 | 1.49 × $10^{-9}$ (88.7) | 8.8 | 95 |

[a] Percent recovery of IFN on the basis of protein amount.

**Table II.** Body Distribution of IFN-Pullulan Conjugate or Other Agents After Their IV Injection to Mice

| | Percent Radioactivity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Free IFN | | | IFN-pullulan Conjugate | | | Free IFN + Pullulan | | |
| Organ | 1 hr | 3 hr | 24 hr | 1 hr | 3 hr | 24 hr | 1 hr | 3 hr | 24 hr |
| Blood | 6.43 ± 1.70[a] | 1.35 ± 0.56 | 0.14 ± 0.08 | 9.11 ± 1.67 | 2.67 ± 0.78 | 0.53 ± 0.14 | 6.30 ± 2.02 | 1.99 ± 0.56 | 1.06 × 0.08 |
| Heart | 0.10 ± 0.03 | 0.08 ± 0.02 | ND | 0.13 ± 0.02 | 0.06 ± 0.03 | ND | 0.05 ± 0.01 | 0.02 ± 0.01 | ND |
| Lung | 0.14 ± 0.02 | 0.19 ± 0.09 | 0.03 ± 0.01 | 0.40 ± 0.09 | 0.17 ± 0.06 | 0.03 ± 0.00 | 0.23 ± 0.10 | 0.08 ± 0.02 | ND |
| Thymus | 0.04 ± 0.01 | 0.03 ± 0.01 | ND | 0.08 ± 0.04 | 0.06 ± 0.02 | ND | 0.04 ± 0.01 | ND | ND |
| Liver | 1.04 ± 0.25 | 0.86 ± 0.22 | 0.19 ± 0.07 | 37.6 ± 3.84 | 28.5 ± 3.78 | 23.0 ± 3.21 | 2.28 ± 0.32 | 1.02 ± 0.06 | 0.17 ± 0.06 |
| Spleen | 0.21 ± 0.09 | 0.24 ± 0.09 | 0.16 ± 0.03 | 2.56 ± 0.39 | 1.03 ± 0.26 | 1.02 ± 0.25 | 0.30 ± 0.12 | 0.34 ± 0.09 | 0.09 ± 0.03 |
| Kidney | 1.25 ± 0.06 | 0.88 ± 0.25 | 0.05 ± 0.02 | 1.57 ± 0.45 | 0.55 ± 0.07 | 0.12 ± 0.03 | 1.40 ± 0.49 | 0.81 ± 0.16 | ND |
| Gastrointestinal tract | 22.2 ± 3.43 | 15.6 ± 6.43 | 4.20 ± 1.28 | 19.6 ± 5.20 | 7.63 ± 2.07 | 1.80 ± 0.27 | 19.4 ± 3.85 | 14.5 ± 5.20 | 6.20 ± 2.02 |
| Thyroid gland | 0.07 ± 0.04 | 1.45 ± 0.67 | 1.56 ± 0.78 | 0.05 ± 0.01 | 0.80 ± 0.50 | ND | 0.07 ± 0.01 | 0.12 ± 0.02 | 0.10 ± 0.01 |
| Carcass | 26.5 ± 5.79 | 26.1 ± 9.25 | 21.6 ± 4.45 | 16.8 ± 5.20 | 14.5 ± 3.35 | 12.0 ± 2.47 | 28.2 ± 4.31 | 24.0 ± 4.02 | 23.4 ± 2.90 |
| Excretion | 33.7 ± 5.98 | 49.6 ± 14.1 | 63.9 ± 12.5 | 10.7 ± 3.67 | 18.8 ± 4.01 | 49.2 ± 6.08 | 33.0 ± 8.56 | 49.3 ± 8.04 | 62.3 ± 12.2 |

Note: ND; Not detected.
[a] Mean ± SE.

corresponding conjugate when evaluated using $^{125}$I-labeled IFN. On the other hand, the amount of pullulan recovered was 95% of the theoretical value, irrespective of the reaction conditions. Approximately 10% of IFN activity was retained in the pullulan conjugate when the antiviral activity before and after pullulan conjugation was compared.

### Body Distribution of IFN-Pullulan Conjugate After IV Injection

The body distribution of IFN was considerably changed by its pullulan conjugation as is apparent from the comparison of free IFN and IFN + pullulan (Table 2). Liver accumulation of IFN significantly increased through pullulan conjugation while the accumulation in the gastrointestinal tract and carcass was reduced. The enhanced accumulation tended to decrease with time but was observed even 24 hr after injection, in contrast to free IFN. The simple mixing of pullulan did not affect the distribution profile of IFN, indicating that the pullulan conjugation is essential to enhance the liver accumulation of IFN.

### 2-5A Synthetase of Mouse Organs After IV Injection of IFN-Pullulan Conjugate

Figure 2 indicates that iv injection of free IFN induced both the 42-kD 2-5A synthetase and the 30-kD protein in a dose-dependent manner, irrespective of the organ type, although the induction level of the liver and spleen was somewhat higher than that of the lung. Although a similar effect on the induction was observed for the IFN-pullulan conjugate, the organ profile of the two protein induction was different from that of free IFN injection. Pullulan conjugation enabled IFN to enhance the level of both 42-kD and 30-kD proteins in the liver to a great extent compared with that in other organs. In addition, 2-5A synthetase was hardly induced in mice injected with IFN-free pullulan alone, irrespective of the organ type.

Figure 3A and 3B show the dose dependence of 2-5A synthetase induction in the liver and its duration, respectively. The 42-kD 2-5A synthetase and the 30-kD protein were induced by free IFN injection in a dose-dependent manner. The injection

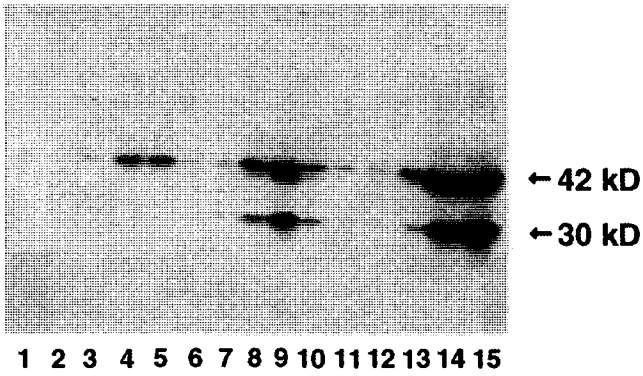


**Fig. 2.** Western blotting analysis of the 2-5A synthetase in the liver, spleen, and lung of mice receiving the iv injection of IFN-pullulan conjugate or free IFN. On one day after the injection tissue lysates were prepared from the organs of mice injected by PBS (lanes 1, 6, and 11), pullulan (lanes 2, 7, and 12), $10^3$ IU of free IFN (lanes 3, 8, and 13), $10^4$ IU of free IFN (lanes 4, 9, and 14), or IFN-pullulan conjugate containing $10^3$ IU IFN (lanes 5, 10, and 15). Lung: lanes 1–5; Spleen: lanes 6–10; Liver: lanes 11–15.

of IFN-pullulan conjugate enhanced the level of 42-kD 2-5A synthetase to a greater extent than that of free IFN, although the IFN dose was much lower than that of free IFN. For example, the level of 2-5A synthetase induced by the conjugate with $10^3$ IU of IFN was higher than the highest level induced by free IFN ($10^6$ IU). The induction level by the conjugate was retained at a high level over 3 days and decreased on Day 4. In contrast, the induction level by free IFN was rapidly lost within the first day (Figure 3B).

## DISCUSSION

The present study demonstrates that chemical conjugation of IFN with pullulan is effective in targeting IFN to the liver and consequently exerting its antiviral activity therein. This can be explained in terms of a high affinity of pullulan for the liver.

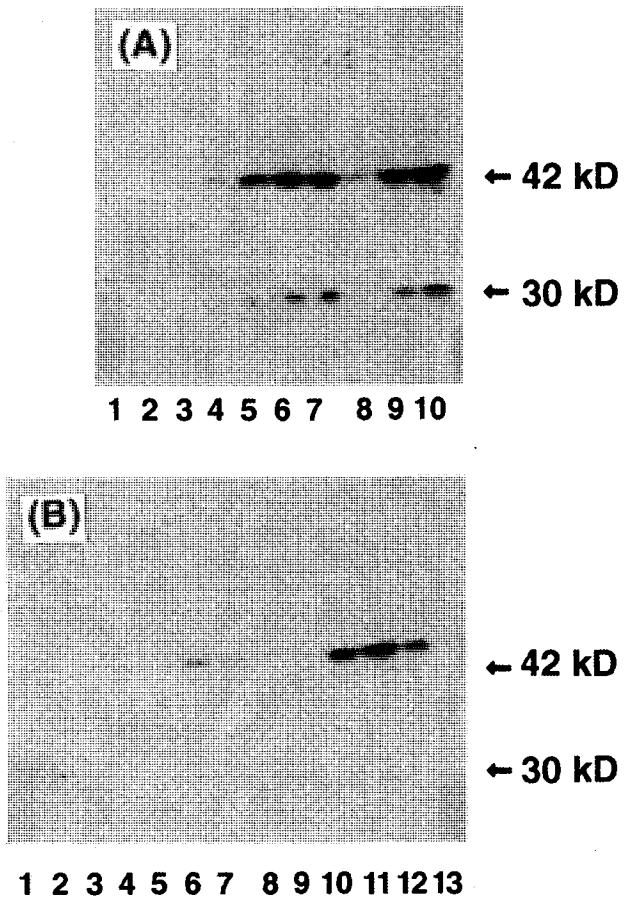


**Fig. 3.** (A) Dose-response of 2-5A synthetase activity of liver one day after injection. Tissue lysates were prepared from the liver of mice injected by PBS (lane 1), pullulan (lane 2), $10^2$ IU of free IFN (lane 3), $10^3$ IU of free IFN (lane 4), $10^4$ IU of free IFN (lane 5), $10^5$ IU of free IFN (lane 6), $10^6$ IU of free IFN (lane 7), IFN-pullulan conjugate containing 10 IU IFN (lane 8), IFN-pullulan conjugate containing $10^2$ IU IFN (lane 9), and IFN-pullulan conjugate containing $10^3$ IU IFN (lane 10). (B) The 2-5A synthetase activity of the liver 1 day (lanes 2, 6, and 10), 2 days (lanes 3, 7, and 11), 3 days (lanes 4, 8, and 12), and 4 days after injection (lanes 5, 9, and 13). Tissue lysates were prepared form the liver of mice injected by PBS (lane 1), $10^3$ IU of IFN (lanes 2, 3, 4, and 5), $10^4$ IU of IFN (lanes 6, 7, 8, and 9), and IFN-pullulan conjugate containing $10^3$ IU IFN (lanes 10, 11, 12, and 13).

Pullulan is edible and has been extensively used for food and pharmaceutical additives. We have already demonstrated that pullulan tends to accumulate to the liver to a significant extent compared with other water-soluble polymers, such as poly(ethylene glycol), poly(vinyl alcohol), and dextran (6,11). In the present study, we used pullulan with the molecular weight of 200,000 to conjugate IFN since the molecular weight higher than 100,000 did not further increase accumulation in the liver (6).

Unlike other organs, the liver vasculature is comprised of discontinuous blood vessels which allow most water-soluble substances to penetrate through the vascular walls (12), resulting in their high accumulation in the extravascular tissue of the liver. Thus, it is likely that the high affinity of polysaccharides, especially pullulan, for the liver may be due to their own inherent nature. Liver cells are reported to possess lectin-like asialoglycoprotein receptors which have a biological affinity for sugar residues (13). It has been demonstrated that introduction of sugar residues into water-soluble polymers enhances their liver accumulation (4,5).

As revealed above, the body distribution study demonstrated that the liver accumulation of pullulan was comparable to that of macromolecules bearing sugar moieties. It seems reasonable to suggest that a specific interaction of some liver receptors with pullulan leads to high accumulation in the liver since this polymer is comprised of repeated sugar units. Dextran was accumulated in the liver to a less extent than pullulan although they are both polysaccharides (6). The liver receptors must recognize the difference between the two polysaccharides in their sugar unit as a binding site. A study on the cellular localization of pullulan accumulation between parenchymal and nonparenchymal cells is presently underway. Introduction of cationic residues to dextran also exhibited enhanced liver accumulation, probably due to the ionic interaction of the cationized dextran with the negatively charged liver cells (3). This presumed mechanisim for liver accumulation of cationic macromolecules is different from the biospecific interaction of nonionic pullulan with the liver receptors.

2-5A synthetase, an enzyme or protein specifically induced by IFN, seems to play an important role in antiviral, antiproliferative, and immunomodulating activities of IFN (14). Induction of this 2-5A synthetase in a certain organ implies an efficient exertion of the antiviral function of IFN therein. Iv injection of IFN-pullulan conjugate was more efficient than that of free IFN with respect to the IFN amount required for the induction of 2-5A synthetase. This should be ascribed to the fact that pullulan conjugation enabled IFN to accumulate in the liver. The minimum amount of IFN required for the antiviral activity induction by the IFN-pullulan conjugate was $10^2$ times or more as low as that required for induction by free IFN. It should be noted that the conjugation process reduced the antiviral activity of IFN by 1/10. This activity loss can be explained in terms of protein denaturation and the steric hiderance of pullulan. It is possible that a chemical reaction irreversibly alters the conformational structure of the proteins, resulting in their denaturation. Secondly, pullulan molecules bound to IFN may impair its receptor binding ability, which reduces its biological activity. However, even if this activity loss is taken into consideration, the conjugate still showed a high efficacy in enhancing the activity induction of liver 2-5A synthetase.

The 2-5A synthetase induced by the IFN-pullulan conjugate was retained at a high level for a longer time period than that induced by free IFN. This is probably due to prolonged liver accumulation of IFN through pullulan conjugation (Table 2). It is conceivable that a prolonged retention of large-sized pullulan in the extravascular space of liver and additionally that its interaction with lectin receptors of liver cells will enable IFN molecules to concentrate on the cell surface because several IFN molecules are conjugated to one pullulan molecule. It has been recognized that binding of one IFN molecule to its cell surface receptor leads to the initiation of subsequent intracellular responses (15). If IFN molecules in the free form bind to the receptor, the intracellular response will be initiated while they are internalized into the cell to be metabolized. On the other hand, IFN molecules being bound to the conjugate will also bind to the receptor but the subsequent internalization and metabolization will

be highly suppressed because of their immobilization to pullulan. It is possible that IFN molecules are released from the receptor without internalization and again bound to other receptors. Such concentration and re-use effects should enable IFN to prolong period of 2-5A synthetase induction.

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