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Induction of Human Leukocyte Interferon by Heat-Treated Poly I: Poly C

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Synthetic double stranded RNA (poly I: poly C) was prepared from polyinosinic acid (poly I) and polycytidylic acid (poly C) by heat-treated followed by gradual cooling, and was used for induction of human leukocyte interferon (IFN). When poly I: poly C solution was heated at 37 - 65°C for 30 minutes, high activity of human IFN (10,000 - 60,000 i. u./ml) was obtained. In this system, the optimum molecular weight of poly I: poly C to induce IFN was 12.6 - 17.6 S. The properties of induced IFN were heat- and acid- stable, and it was neutralized with anti-IFN α serum. So, it was confirmed that it was IFN α.

Human leukocyte interferon, which is already applied to clinical trial, has been ordinarily induced by viruses, such as HVJ (Sendai virus) or NDV (Newcastle disease virus) (1,2). While these viruses are active inducers of human leukocyte IFN, they are hard to handle on a large scale in blood centers. With regard to purification of IFN, the contamination of heterogenious proteins such as virus- proteins and ovalbumin (viruses are multiplied in eggs) is a serious problem. Also, in the case of gene-engineering technique, the contamination of proteins of E.coli can not be ignored. The development of synthetic chemical inducers, which are easy to handle and stable under any conditions, is indispensable for large-scale production of human leukocyte IFN.

Synthetic double stranded RNA (poly I: poly C) has been ordinarily used to induce human fibroblast IFN (3). However, it has been reported that poly I : poly C is not an effective inducer of human leukocyte IFN (4). In this report, we show that heat-treated poly I: poly C act as an effective inducer to induce human leukocyte IFN. Actually, the poly I: poly C, prepared by suitable heat-treatment (37 - 65°C) induced a large amount of leukocyte IFN (10,000 -60,000 i.u./ml of IFN in culture fluid).

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MATERIALS AND METHODS

Chemicals: Polyinosinic acid (poly I) and polycytidylic acid (poly C) were purchased from Yamasa Shoyu Co. Ltd.(Japan). Ficoll was purchased from Farmacia Fine Chemicals (Sweden).

Preparation of double stranded poly I: poly C: Equal moles of poly I and poly C were mixed in phosphate buffered saline (Ca^{2+}, Mg^{2+} free) (PBS (-)) to make concentration of 4 mg/ml. These mixed poly I and poly C were heated at 37 - 120°C for approximately 30 minutes, then gradually cooled to form double strand. These poly I: poly C were stored at 4°C until use.

Ultracentrifugation: Sedimentation analysis were performed with a MOM MODEL 3170/b (Hungarian Optical Works). Sedimentation velocity was determined at 60,000 rpm, and the values were corrected to $S^{0}_{20,w}$. Measurement was made on solution of poly I: poly C in concentration of 0.01% - 0.02% in PBS(-) between 20 - 30 °C.

Ultra-Violet spectra and Circular-Dichroism spectra: UV-spectra were analyzed with CARY 219 spectrophotometer. CD-spectra were analyzed with spectropolarimeter (Japan Spectroscopic Co. Ltd.). Measurement was made on solution of poly I: poly C in concentration of 0.01% in PBS(-).

Cells, Virus and medium: Mononuclear leukocyte cells (MNL) were prepared from human peripheral whole blood cells by the methods of Ficoll-hypaque gradient as described elsewhere (5). FL cells and Sindvis virus were obtained from Drs. Khono and Kohase (National Institute of Health of Japan). FL cells were maintained with minimum essential medium supplemented with 5% calf serum.

Interferon induction: MNL were suspended in RPMI 1640 medium containing 10 mM HEPES and 2% human plasma at a concentration 2×10^{7} cells/ml. Pre-incubation of cells were performed by stirring for 2-3 hrs at 37°C, then poly I: poly C were added to the culture at a concentration of 200 μg/ml. The culture was stirred for additional 20 - 24 hrs at 37°C. After cells were precipitated by centrifugation, supernatant fluid was harvested and stored at - 80°C until assay.

Interferon assay: Interferon in culture fluid was titrated by the cytopathic inhibition microassay methods (6), using FL cells and Sindvis virus as challenge virus. The titers are given in international reference standard units, using NIH standard of human leukocyte interferon as a reference .

Antiserum: Anti- human IFN β serum was obtained from Drs. Shimizu and Ozawa (Toray Industries, Inc.). Anti-human IFN α serum was obtained from S. Mitsunaga (Central Blood Center, The Japanese Red Cross).

Polyacrylamide gel electrophoresis: Crude IFN was electrophoresed on sodium dodesylsulfate (SDS)-polyacrylamide gels (6.25 - 10 %), according to the method of Laemmli (7). The gels were sliced at 2 mm of thickness and anti-viral activities of each slices were eluted in RPMI 1640 medium and assayed as described above.

RESULTS

Five preparations of poly I: poly C ($S^{0}_{20,w}$= 12.6) which were prepared by heating at different temperatures (no-treatment, 37°C, 65°C, 95°C, 125°C) were examined for their IFN inducing activities in human leukocyte cells. (Table 1) shows that maximal amount of IFN was obtained when using poly I: poly C which

Table 1 IFN inducing activities of poly I: poly C which were prepared by heating at different temperatures.
Equal moles of poly I (S=6.9) and poly C (S=6.5) were mixed in PBS(-) (4 mg/ml) and heated at described temperature followed by gradual cooling. MNL ($2x10^7$ cells/ml) were induced by these poly I: poly C (200 μg/ml).
* ; autoclaved for 20 minutes.
The presence of poly I: poly C in IFN preparations does not influence on IFN assay.

Treatment	IFN titer (u/ml) Exp.1	Exp. 2	Exp. 3	Exp. 4
none	3,000	2,400	1,200	5,000
37°C	>50,000	—	—	40,000
65°C	>50,000	36,000	33,000	36,000
95°C	40,000	—	—	8,000
120°C*	<60	<60	<60	<60

were heated at 37°C and 65°C. These high active poly I: poly C showed different patterns of UV and CD spectra from inactive poly I: poly C (no-treatment,120°C). With regard to UV spectra, high active poly I: poly C (37°C, 65°C) showed high level of hypochromicity, and differed from inactive poly I: poly C (no-treatment, 120°C) which showed lower level of hypochromicity as shown in (Fig.1). In the case of CD spectra, the increase of the peak at 220 - 250 nm, and the decrease of the peak near 275 nm, suggested new base-pairing of double strand and "stacking" of base to base in high active poly I: poly C (37°C, 65°C). At high temperature (120°C) treatment, a part of poly I: poly C strand might be destroyed and turn to loose conformation. Like this, it might be suggested that the conformation of double stranded poly I : poly C and its IFN inducing activity are closely related. Stable and highly orderly double stranded poly I: poly C act as an effective human leukocyte IFN inducer.

Dose response curve was examined by using poly I: poly C which was heated at 40°C. MNL were induced by several doses of poly I: poly C as described

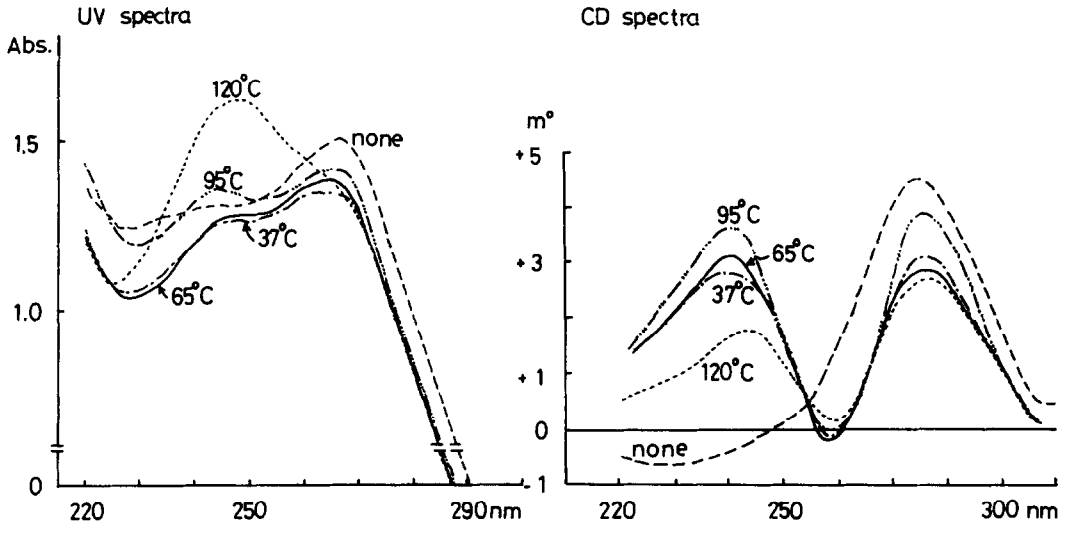

Fig.1 Poly I: poly C (12.6 S) prepared by heat-treatment at indicated temperature were diluted in PBS(-) at a concentration of 0.01 %. UV and CD spectra were analyzed at a range of 220 - 300 nm.

above. As shown in (Fig.2), maximal amount of IFN was obtained at 200 μg/ml of poly I: poly C.

Next, IFN inducing activities of various sized poly I; poly C were examined. (Table 2) shows that 10.4 S poly I: poly C always less active than the others,

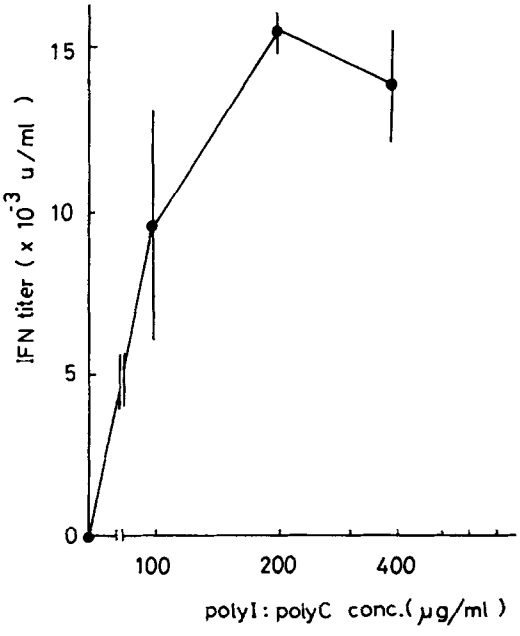

Fig.2 Dose-response of poly I: poly C
MNL were induced by several doses of poly I: poly C (S=12.6, heated at 40°C). MNL concentration was 2×10^7 cells/ml.

Table 2 IFN inducing activities of various sized poly I: poly C. Each pairs of poly I: poly C were mixed and heated at 40°C and gradually cooled. MNL were induced by these poly I: poly C (200 μg/ml of poly I: poly C, MNL concentration was $2X10^7$ cells/ml).

$S^0_{20,w}$ of	$S^0_{20,w}$ of		IFN titer (u / ml)				
Poly I : Poly C	Poly I	Poly C	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
10.4	4.2	5.3	6,700	5,000	4,000	6,600	21,000
12.6	6.9	6.5	26,700	44,700	24,000	13,200	29,700
17.6	8.9	8.9	27,200	39,800	11,000	20,900	59,200
24.2	10.7	11.0	23,700	20,000	9,000	5,300	37,300

and 12.6 S and 17.6 S poly I: poly C constantly induced a large amount of IFN compared to 24.2 S poly I: poly C. The results differed from previous reports in fibroblast cells and in mice, in which systems the IFN amount depends upon the molecular size of poly I: poly C, and the larger molecular sized poly I:

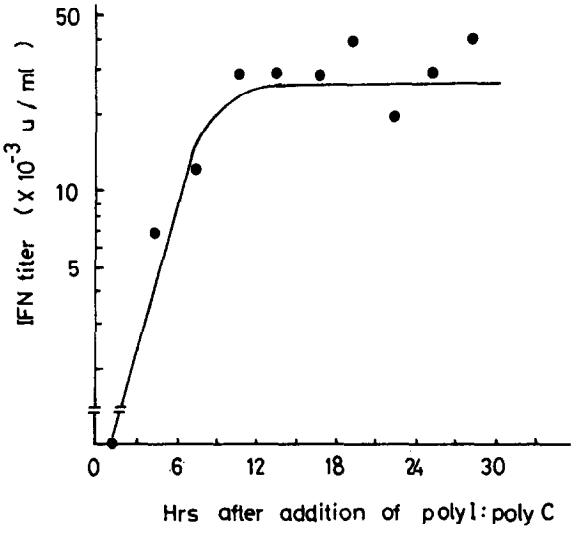

Fig. 3 Time course of IFN appearance in culture fluid MNL ($1x10^7$ cells/ml) were induced by poly I: poly C (S=12.6, heated at 40°C). Crude IFN in culture fluid was harvested at indicated time and assayed as described in Materials and Methods.

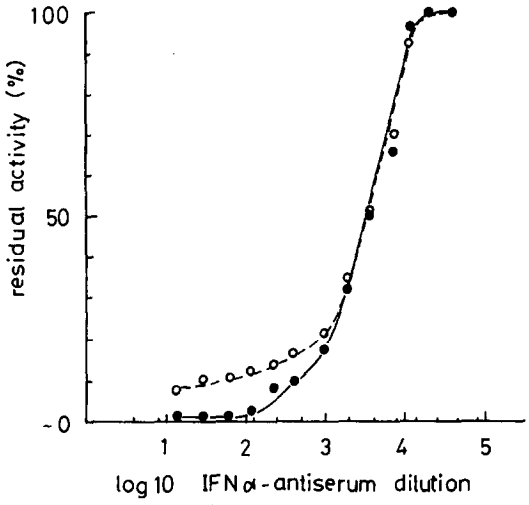

Fig.4 Neutralization with anti-IFN α serum.
100 u of IFN were neutralized with anti-serum for 30 minutes at 37°C and 1 hr at 4°C. Residual activities were measured as described in Materials and Methods. The activity of the sample which was not neutralized was 100%.
--o-- poly I: poly C induced IFN
—●— HVJ induced IFN
HVJ induced IFN; MNL (1×10^7 cells/ml) were induced by HVJ (150 HAU/ml) for 24 hrs and virus was killed with UV.

poly C (> 20 S) induce larger amount of IFN (8,9). By Trypane-blue exclusion test, cellular toxicity of poly I: poly C was not detected after 24 hrs incubation with each size of poly I: poly C (90 - 95 % of lymphocytes survived after 24 hrs incubation).

The IFN appeared in culture fluid at 3hrs after addition of poly I: poly C and saturated at 12 hrs (Fig.3). This time course well corresponds to HVJ induced human leukocyte IFN appearance in culture fluid (1).

The poly I: poly C induced IFN was acid (pH 2)- and heat (56°C)-stable (data not shown) and was neutralized with anti-human IFN α serum (Fig.4), but not neutralized with anti-human IFN β serum. From these results including the data of time course, it was concluded that poly I: poly C induced IFN was IFN α.

Molecular weight of the IFN was analyzed by SDS-PAGE. As shown in (Fig.5) the molecular weight of the IFN was 15,000 - 20,000 and closely corresponded to HVJ induced IFN (10).

Neither priming (11) nor super-induction (12) (the former was applied to HVJ induced leukocyte IFN; the latter was applied to poly I: poly C induced

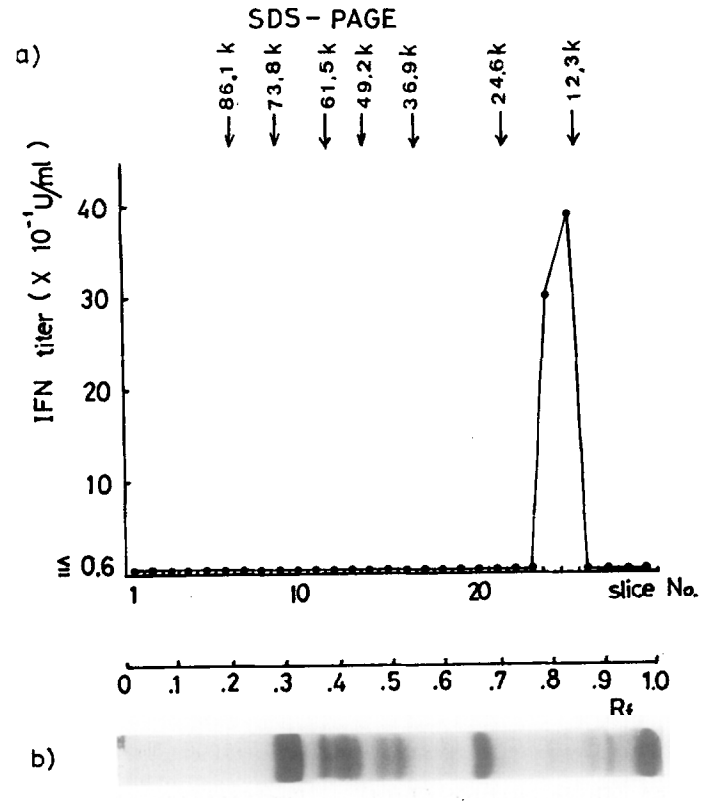

Fig.5 SDS-PAGE analysis of crude IFN
Crude IFN (16,000 u/ml) were dialysed against distilled water at 4°C and electrophoresed on SDS-PAGE as described in Materials and Methods.
a) The antiviral activities of each slices.
molecular marker were; cytochrome C monomer 12.3 K, dimer 24.6 K, trimer 36.9 K, tetramer 49.2 K, pentamer 61.5 K, hexamer 73.8 K, heptamer 86.1 K,
b) Thymol blue stained pattern.

fibroblast IFN) was effective in human leukocyte cells (MNL) when induced by poly I: poly C (data not shown). Moreover, the presence of DEAE-dextran (Farmacia, MW 500,000) had no effects on IFN inducing activities of poly I: poly C (data not shown). This result also differed from the case of fibroblast system, in which DEAE-dextran is often effectively used (13).

DISCUSSION

We found that heat-treated poly I: poly C is effective for a induction of a high amount of leukocyte IFN. Between the active and inacvive forms of poly I: poly C, there were seen differences in the patterns of UV and CD spectra. It is suggested that the conformation of double strand is important to induce IFN in human leukocyte. De Clercq described (14) that heat-activated poly

r(A-U) showed a greater affinity for the cell and persisted for a longer time at the outer cell membrane of human skin fibroblast cell than non-activated poly r(A-U). Also in the case of our system, suitable active conformation of double stranded poly I: poly C could be obtained by heat-treatment and gradual cooling. It is not clear as yet whether this active double stranded poly I: poly C enters into the cells or merely comes into contact with outer cell membranes.

As regards priming, which is very useful for inducing greater amount of human leukocyte IFN when induced by HVJ, it was not effective when induced by poly I: poly C. The process of induction may be different between poly I: poly C-incuction and HVJ-induction. This difference in the process of induction may result in the difference of heterogeniety of IFN between poly I: poly C induced and HVJ induced IFN.

Our induction system(poly I: poly C — human leukocyte) could be applied to large-scale production, and we could obtain a large amount of IFN (10,000 - 40,000 i.u./ml of IFN in culture fluid). Purification of IFN on a large-scale will be more simplified than in the case of HVJ induced IFN.

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REFERENCES

1) Morgensen, K. E., and Cantell, K. (1977) Pharmac. Ther. C. 1 369- 381
2) Matsuo, A., Hayashi, S., Kishida, T. (1974) Japan J. Microbiol. 18 21-27
3) Knight, E. Jr. (1976) Proc. Natl. Acad. Sci. USA. 73 520- 524
4) De Clerq, E. (1977) Texas Rep. Biol. Med. 35 29- 38
5) Harris, R. and Ukaejiofo, E. O. (1970) Brit. J. Haematol. 18 229-235
6) Ho, M., and Enders, J. F. (1959) Proc. Natl. Acad. Sci. USA. 45 385-392
7) Laemmli,U. K. (1970) Nature 227 680-685
8) Machida, H., Kuninaka, K., Yoshino, H. (1976) Japan J. Microbiol. 20 71-76
9) Ishida, N., and Suzuki, F. (1974) Saishin-igaku 29 631-642
10) Rubinstein, M., Rubinstein, S., Familletti, P. C., Miller, R. S., Waidam, A. A. (1979) Proc. Natl. Acad. Sci. USA. 76 640-644
11) Cantell, K. (1970) Sym. Series Immunobiol. Standard 14 6-9
12) Vilcek, J., and Ng, N. H. (1971) J. Virol. 7 588-594
13) Pitha, P. M., and Carter, W. A. (1971) Virology 45 677-681
14) De Clerq, E., Wells, R. D., and Crant, R. C. (1971) J. Mol. Biol. 56 83-100