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PRODUCTION AND CHARACTERIZATION OF INTERFERON INDUCED IN CHICKEN LEUKOCYTES BY CONCANAVALIN A

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Summary. — Interferon (IFN) was induced in chicken peripheral blood leukocytes by exposure to Concanavalin A (Con A). It has reached a maximum level 96 hr after stimulation with the optimum dose of 10 μg/ml. The IFN was partially purified by chromatography on controlled-pore glass adsorbent and was charaterized as a fairly acid-stable and heat-resistant, trypsin-sensitive and species-specific substance with M_r of 20,500 Da. Antiviral response by this type of IFN in chick fibroblast culture developed within several hours. This study provides first evidence of the presence of IFN in supernatants of mitogen-stimulated chicken peripheral leukocytes.

Key words: chicken leukocyte; Concanavalin A; interferon induction

Introduction

For a number of years we have been studying the mechanism of IFN induction by different human adenovirus types in chick fibroblasts and chicken leukocytes (Béládi *et al.*, 1979). The intriguing problem of comparing the viral and mitogen IFN induction in an avian system prompted us to produce mitogen-induced chicken IFN. In this communication we present a method for the induction of IFN production in chicken peripheral blood leukocytes by Con A including some physicochemical and biological properties of this type of IFN.

Materials and Methods

Cells and cultures. Peripheral blood mononuclear cells were isolated from heparinized blood from 5—7 month-old chickens of the White Leghorn strain by low-speed centrifugation (Hudson and Roitt, 1973), followed by discontinuous density gradient centrifugation on a Ficoll-Uromiro gradient (Böyum, 1979). The cells were washed three times in phosphate-buffered saline (PBS). The leukocytes obtained were suspended in RPMI 1640 medium supplemented with 5% foetal calf serum (FCS) 2 mmol/l glutamine, 100 U/ml penicillin and 50 μg/ml gentamycin. Cell viability was monitored by the exclusion of 0.2% trypan blue, and the cell number was adjusted to the appropriate dilution. Cells were cultured in flat-bottom microtitre tissue culture plates (Linbro, Greiner) and incubated at 37 °C in 5% CO_2/air or spinner culture at 37 °C. Other cells used in this study were: secondary chick embryo fibroblast, mouse L_{929}, bovine MDBK and human WISH cells. They were propagated and maintained as monolayers in Eagle's minimal essential medium (MEM) with 5 or 10% FSC, 100 U/ml penicillin and 100 μg/ml streptomycin.

Virus. The Indiana serotype of vesicular stomatitis virus (VSV) was used as challenge virus.

Production of IFN. IFN was induced in the leukocyte suspension in microplate cultures by exposure to Con A (Calbiochem-Boering Corp., La Jolla, Calif.) under a variety of cell culture conditions. In the spinner culture, IFN production was induced by the addition of 10 μg/ml Con A. The cell suspension was then incubated at 37 °C. After 96 hr, supernatant fluid was collected and clarified by centrifugation at 1 500 rev/min for 5 min and stored at −20 °C.

IFN assay. The antiviral activity in culture supernatants was determined by cytopathic inhibition assay in microtitre plates, with secondary chick embryo fibroblast (10^5 cells/well) as indicator cells. Serial twofold dilutions of IFN were made in duplicate in microwells, and after overnight incubation the cells were challenged with VSV (10^5 PFU/ml). Titrated sample of virus-induced IFN laboratory standard was included in the assay in addition to the IFN samples tested. The IFN titre was calculated as reciprocal of the dilution in the well in which 50% of the monolayer was protected from the cytopathic effect (CPE) of the virus. The reference IFN titre was determined and the end-point of the samples was adjusted to absolute unites. Since no internationally accepted reference preparation was available for mitogen-induced IFN, the titre of IFN was expressed as chick IFN MRC Research Standard A 62/4 (Mill Hill, London) units (U) per millilitre.

Purification and molecular mass determination of IFN. The chromatographic procedure will be described in detail elsewhere (Taródi et al., manuscript in preparation). Briefly, the crude IFN preparation was loaded onto a controlled-pore glass (CPG 10, pore size 75 Å, 120—200 mesh, Electro-Nucleonics, Fairfield, N. J., U.S.A.) column, which was equilibrated with PBS, pH 6.8, until negligible absorbance was measured by the UV light monitor at 280 nm. IFN was then eluted with 0.5 mol/l Tris-HCl buffer, pH 8.0, containing 1.5 mol/l NaCl. This procedure resulted in a tenfold concentration of the starting material, with a considerable purification. Fractions were collected and titrated for antiviral activity. The peak fractions were pooled and concentrated on an ultrafilter having a nominal molecular mass limit of 10,000 Da. The partially purified and concentrated IFN samples were stored at −20 °C for several months without considerable loss of activity. Further purification was carried out by Ultrogel AcA-54 chromatography, and then washed with the same buffer. Fractions were collected and analysed for antiviral activity. The following internal markers with known M_r were used: bovine serum albumin (67.000), ovalbumin (45,000) and trypsin inhibitor (21,500).

Characterization of IFN. The thermal stability of CPG-purified IFN was examined by incubating parallel samples in sealed ampoules (Flow Lab) in a water-bath at 60, 70, 80 or 90 °C for various time intervals, followed by quick cooling on ice.

The acid stability was examined through dialysis of crude and GPG-purified preparations against 0.05 mol/l HCl/KCl buffer, pH 2.0, for 24 hr at 4 °C, followed by similar dialysis against PBS, pH 7.2.

Inactivation by trypsin was determined by incubation of the preparations in the presence of 1 mg/ml crystalline trypsin (Worthington) for 1 hr at 37 °C, followed by the addition of 2 mg/ml soybean inhibitor (Serva).

The host range of the crude and CPG-purified products was examined by titration on cell cultures of different animal species (mouse L_{929}, bovine MDBK and human WISH cells).

Measurement of the antiviral response. Cultures of confluent secondary chick embryo fibroblasts were prepared in microtitre plates. Cells were treated with serial twofold dilutions of Con A- or virus-induced chicken leukocyte IFN (200 U/ml) in Eagle's MEM supplemented with 5% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. At different intervals the IFN-containing medium was removed and the cells were rinsed four times with PBS. The cells were challenged with VSV (1 PFU/cell) in Eagle's MEM and incubated at 37 °C for 16 hr or until 100% CPE was noted in the virus control wells. All media and washing fluids were prewarmed to 37 °C. The inhibition of the CPE in cells treated with IFN for different times was expressed as a percentage of inhibition provided by a standard IFN preparation of the known titre, included in each assay. The IFN titre observed in cells treated with IFN for 24 hr represented 100% inhibition.

Results

Optimum conditions for production of IFN

In order to develop a suitable method for the production of mitogen induced chicken IFN, experiments were carried out under a variety of cell

Table 1. Acid (pH 2)-stability of Con A-induced chicken leukocyte IFN

Experiment no.	pH 2 treatment	IFN titre U/ml	Residual activity %
1	—	1433.66 ± 425.05*	44.76 ± 11
	+	641.66 ± 162.48	
2	—	2484.50 ± 487.20	28.68 ± 8
	+	712.50 ± 198.70	
3	—	788.50 ± 47.40	51.45 ± 1
	+	400.50 ± 7.78	

* arithmetric mean of three titrations ± SD

culture conditions. Peripheral blood mononuclear cells were suspended in RPMI 1640 medium or Eagle's MEM with 5 % or 10 % FCS to a density of $1 - 10 \times 10^6$ cells/ml and cultured in flat-bottom microtitre plates. IFN production was induced by Con A (0.5 — 20 μg/ml). The cell suspensions were incubated at 37 or 40 °C. The highest IFN yields were obtained with 10 μg/ml Con A using 10^7 cells/ml in RPMI 1640 medium supplemented with 5 % foetal calf serum and 2 mmol/l glutamine. Fig. 1 shows the kinetics of IFN production in the leukocyte culture after stimulation with 10 μg/ml Con A. A measurable activity started to appear on the third day, reaching the maximum titre on the fourth day. The yields of IFN induced by Con A chicken leukocytes varied within the range from $10 - 10^3$ U/ml, depending on the birds used. No spontaneous IFN production was ever observed in the control leukocyte cultures.

Physicochemical and biological properties of IFN

IFN, partially purified by chromatography on CPG adsorbent, was characterized with regard to sensitivities to acid, heat and trypsin treatments.

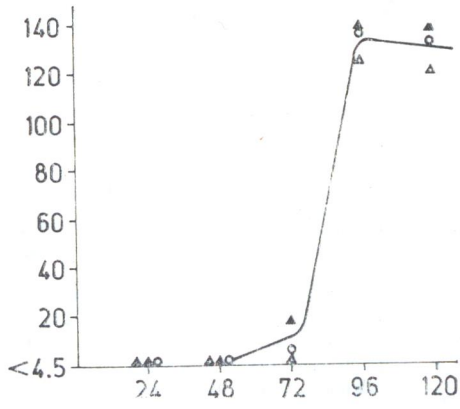

Fig. 1.
Kinetics of IFN production in chicken leukocyte cultures after stimulation with Con A
Each symbol corresponds to one experiment.
Abscissa: time (hr); ordinate: IFN titre (units/ml).

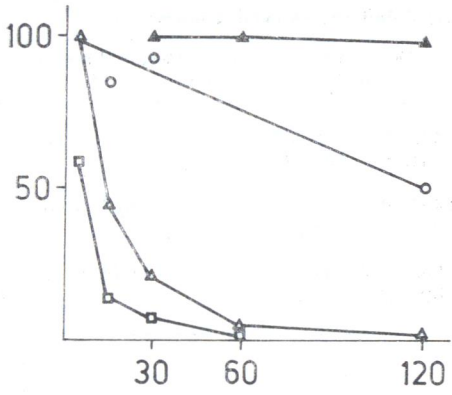

Fig. 2.
Rate of thermal inactivation of CPG-purified Con A-induced IFN
60 °C (▲———▲), 70 °C (○———○), 80 °C (△———△) and 90 °C (□———□)
Abscissa: time (min); ordinate: residual activity (%).

IFN preparations treated at pH 2.0 for 24 hr showed a 30 — 50 % loss of antiviral activity (Table 1), but crude IFNs were acid-stable. The activities of IFN samples were tested after different periods of incubation at 60, 70, 80 or 90 °C. The IFN appeared fairly stable at 60 °C for 2 hr (Fig. 2). At 70 °C the activity decreased gradually. The inactivation curves revealed more rapid inactivation at 80 and 90 °C. After a 60 min heat-treatment, the residual activity was very low. The IFN preparation proved to be trypsin-sensitive.

Ultrogel AcA-54 column chromatography of partially purified IFN showed one peak with an apparent M_r of 20.500 Da (Fig. 3). Crude or CPG-purified preparations were found to be ineffective in inhibition of VSV multiplication in L, MDBK or WISH cells.

The establishment of the antiviral response by Con A-induced IFN was studied in chick fibroblasts including a virus-induced leukocyte IFN sample for comparison. The two types of IFN displayed a similar effect (Fig. 4). The antiviral respons edeveloped gradually, with a maximum activity at 8 hr after treatment with the partially purified Con A- or virus-induced IFN.

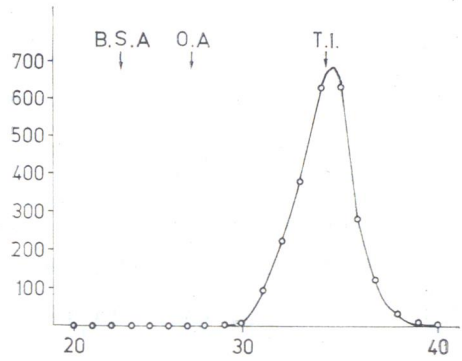

Fig. 3.
Molecular mass determination of mitogen-induced chicken leukocyte IFN by gel filtration chromatography on an AcA-54 Ultrogel column
Molecular mass standards were: bovine serum albumin (BSA), 68,000 Da, ovalbumin (OA) 45,000 Da; trypsin inhibitor (soybean; TI) 21,500 Da.
Abscissa: fraction number; ordinate: IFN titres.

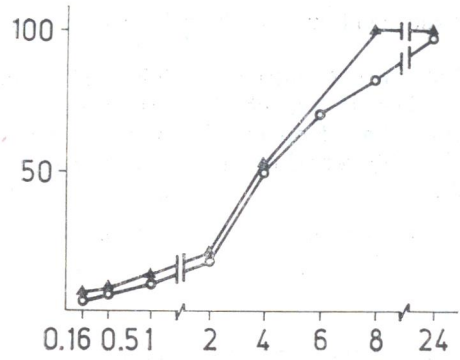

Fig. 4.
Kinetics of antiviral response induction in chick fibroblasts
Cells treated either with Con A-induced IFN (●———●), or with virus-induced chicken leukocyte IFN (○———○). Abscissa: time (min); ordinate: antiviral activity (%).

Discussion

Mitogen-induced IFN production is known in human and mouse systems (Stewart II, 1979). We succeeded in producing IFN in chicken leukocyte cultures stimulated by Con A. Kinetic studies showed that this IFN was produced late in the course of Con A stimulation. The amount of IFN was variable (usually low) compared to other types of chicken IFN. The different activities of the birds could be explained by the high variation in the mitogen response of lymphocyte from chickens not inbred at the major histocompatibility complex (Pink and Miggiano, 1977). The presence of an inhibitor of IFN action could also be responsible for the low activity of the IFN; this has recently been described in the supernatant of a mouse spleen cell culture stimulated by staphylococcal endotoxin A (Lefkowitz and Fleischmann, 1985).

Con A-induced chicken IFN exhibits certain similarities as well as differences in its physico-chemical characteristics compared to those of virus-induced chicken leukocyte and chick fibroblast IFN. Con A-induced IFN appeared as heat-stable as the other two chicken IFNs at 60 and 70 °C, and it was also fairly resistant at 80 °C. Its antiviral activity was not diminished even after 60 min treatment, while the heat-resistant activity of fibroblast and of virus-induced leukocyte IFN represented only 45 and 20 % activities of original IFN preparations ,respectively (unpublished data). Acid-treatment of the Con A-induced IFN resulted in loss of antiviral activity to different degrees, varying from batch to batch. As a control, human immune IFN was also treated in the same experiment; this lost more than 90 % of its activity.

On gel filtration, the antiviral activity of Con A-induced IFN eluted with a protein moiety with an apparent M_r of 20,500 Da. In contrast, the fibroblast and virus-induced leukocyte IFNs have complex molecular compositions, as described earlier (Taródi *et al.*, 1975) and confirmed by the method applied in this study. The host range of Con A-induced IFN was narrow and no activity was detectable on mammalian cells. Chick fibroblasts treated with Con A-induced chicken IFN acquired the antiviral state as

slowly as observed in human and mouse systems with mitogen-induced IFN (Dianzani *et al.*, 1978).

The data observed so far suggest that Con A-induced chicken IFN has many similarities with virus-induced chicken IFNs, which is unique among the IFN systems investigated up to date. Based on our knowledge of Con A-induced chicken IFN, it seems questionable whether in birds gamma-IFN similar to that in mammals exists at all.

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