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SOME BIOLOGICAL PROPERTIES OF THE HUMAN AMNIOTIC MEMBRANE INTERFERON

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Human amniotic interferon was investigated to define the species specificity of its antiviral action and to compare its anti-cellular and NK cell stimulating activities with those of other human interferons. The antiviral effect was titrated in bovine (RV-IAL) and monkey (VERO) cells. Amniotic interferon exhibited, in bovine cells, 5% of the activity seen in monkey cells, while alpha interferon displayed 200%. No effect was detected with either beta or gamma interferon in bovine cells. Daudi cells were exposed to different concentrations of various interferons and the cell numbers were determined. The anticellular effect of the amniotic interferon reached its peak on the third day of incubation. Results suggested a higher activitity for alpha and gamma interferons and a lower activity for beta when compared to amniotic interferon. Using total mononuclear cells as effector cells and K 562 as target cells in a ^{51}Cr release assay, it was demonstrated that low concentrations of amniotic interferon consistently stimulated NK cell activity in cells derived from several donors, the results indicating a higher level of activity with this interferon than with alpha and beta interferons.

Key words: human interferon – amniotic – biological properties

Interferons (IFNs) constitute a family of proteins produced by vertebrate cells. They have many biological properties, among the more prominent of which are their antiviral, anticellular and immunomodulatory activities (Stewart, 1981). They have been used in the therapy of certain viral infections and cancers (Scott & Tyrrell, 1985; Oldham, 1985). For clinical applications, the methodology for its industrial production has been developed, employing leukocytes (Cantell & Hirvonen, 1977), skin fibroblasts (Vilcek et al., 1978); lymphoblastoid cells (Zoon & Buckler, 1977), bacteria (Goeddel et al., 1980) and fungi (Derynck et al., 1983) using DNA recombinant techniques.

Human amniotic membranes produce IFN (Falcoff et al., 1966) in quantities similar to those in leukocytes or skin fibroblasts. The methodology of IFN production from this source is relatively simple and low in cost (Kroon et al., 1990) and it is possible that it could provide enough IFN for treatment of localized diseases by topical application. However, of the biological properties of the human amniotic membrane IFN (IFN-AM), only its antiviral effect has thus far, been reported (Kroon et al., 1990).

In this paper, some of the more relevant biological activities of IFNs – species specificity, anti-cellular effect and NK cell stimulation – are demonstrated for the IFN-AM and compared to the activities of other human IFNs.

MATERIALS AND METHODS

Cells and viruses – Vero cells were obtained from ATCC and cultivated in autoclavable minimum essential medium (MEM) (Yamane et al., 1968) supplemented with 5% sheep serum, antibiotics and glutamine. RV-IAL cells, a line derived from calf kidney in

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the Instituto Adolfo Lutz, São Paulo, SP, Brazil, were grown in Yamane's medium with 10% calf serum, antibiotics and glutamine. Daudi cells, kindly supplied by Dr Sidney Pestka, were cultivated in RPMI 1640 medium with 20% foetal calf serum (FCS) and gentamycin.

Effector cells for the NK assay were obtained by separation of heparinized blood from healthy volunteers using Ficoll-Hypaque density gradient centrifugation. These cells were washed twice in PBS, and resuspended at a concentration of 5 x 10^6 cells/ml. K562 cells, derived from human erythroleukemia, were employed as target cells. Before the assay, these cells were labeled with 150 μCi $Na_2{}^{51}CrO_4$, for 90 min at 37 °C. They were then washed twice in RPMI and resuspended in RPMI containing 5% foetal calf serum at a concentration of 5 x 10^4 cells/ml.

Newcastle disease and Sendai viruses were multiplied in embryonated hen's eggs, encephalomyocarditis virus in L-929 cells and vesicular stomatitis (VSV) virus in VERO cells. Viruses grown in eggs were titrated by estimating the 50% infectious dose for eggs (Reed & Muench, 1938); the others by the 50% infectious dose for VERO cells in microtechnique.

IFN-AM preparations – Interferon was produced essentially as described (Kroon et al., 1990). Briefly, amniotic membranes were separated from human placentas, fragmented, washed and infected with Newcastle disease or Sendai viruses. Medium was then added and collected 24 and 48 h later. The pH was adjusted to pH 2.0 or 3.5. Initially, the medium for the production of IFN-AM contained 1% human plasma; these preparations were concentrated by thrichloroacetic acid precipitation (Gonzaga et al., submitted). With the IFN-AM produced without plasma, the concentration was done by ultrafiltration in YM-10 membranes (Amicon Corporation, Danvers, Mass., USA) and the purification was performed by chromatography in controlled pore glass (CPG) (Reis et al., 1989) applying the concentrated IFN-AM in neutral pH and displacing it with acid pH. If needed, materials were concentrated in dialysis bags by removal of water by polyethylene-glycol.

Interferon titration – Interferons were titrated by a VERO cell-encephalomyocarditis virus microtechnique (Ferreira et al., 1979) using an internal standard. Titers were adjusted accordingly to the average titer of the internal standard. International standards for human IFNs were: alpha interferon – MRC 69/19; beta IFN-NIH G 023-902-527. The titration had an interassay coefficient of variation of 36%. Titers in this paper are expressed in laboratory units. The assay employed is 17.6 more sensitive in relation to the standard alpha IFN and 8 times regarding the standard beta IFN. VSV was employed for challenging RV-IAL cells.

Other human IFNs – Leukocyte IFN was provided by the late Dr Kurt Paucker, Philadelphia, USA with a specific activity of 10^6 IFN units/mg of protein. Beta IFN (Lee Biomolecular Research Laboratories Inc, San Diego, USA) had a specific activity of 10^5 IFN units/mg. Recombinant alpha IFN was supplied by Schering (Intron-A) and it had a specific activity in excess of 2 x 10^8. Gamma IFN was produced in peripheral blood leukocytes induced by phytohemagglutinin and it had a specific activity of 10^2 (Menezes, 1985).

Natural killer cell (NK) assay – Cytotoxicity was determined using the ^{51}Cr release assay (Hiserodt et al., 1985). Briefly, different proportions of effector: target cells were incubated for 4 h in microtiter plates, at 37 °C and 5% CO_2. After the incubation period, 100 ml of supernatant were collected and the radioactivity assessed. The percentage of specific lysis was calculated from the following formula [(experimental release – spontaneous release) + (total label – spontaneous release)] x 100. When IFN was used, effector cells were preincubated with various concentrations of IFN for 45 min at 37 °C. After this, cells were washed twice and used as effector cells in the NK assay.

RESULTS

Species specificity – Human IFNs alpha, beta gamma and IFN-AM were titrated in VERO cells and in RV-IAL cells. A bovine IFN produced in RV-IAL cells (J. R. Santos, personal communication) was also included in the experiment. Results are shown in Table I. The alpha IFN displayed a high degree of cross antiviral activity, having a high titer in bovine cells; human beta and gamma IFNs did not have a measurable titer in bovine cells, showing a clear species-specificity. IFN-AM showed 5% heterologous activity, approximating the behaviour of beta and gamma IFN.

TABLE I

Species specificity of antiviral protection of amniotic and other human interferons in bovine and monkeys cells

IFN Types[a]	Interferons Titer U/ml (x10^{-3}) RV-IAL	VERO	Cross Protection[b] %
Amnion	0.80	16	5
Alpha	64	32	200
Beta	< 0.05	48	< 0.1
Gamma	< 0.05	1.92	< 2.6
Bovine	> 12.80	2.40	< 18.7

a: alpha – international standard (69/19 MRC);
amnion – amniotic membrane IFN induced by Sendai virus;
beta – international standard (G-023.902.527);
gamma – induced by phytohemagglutinin;
bovine IFN – induced in RV-IAL cells by Newcastle disease virus
b: ratio of titer in RV-IAL and VERO cells x 100.

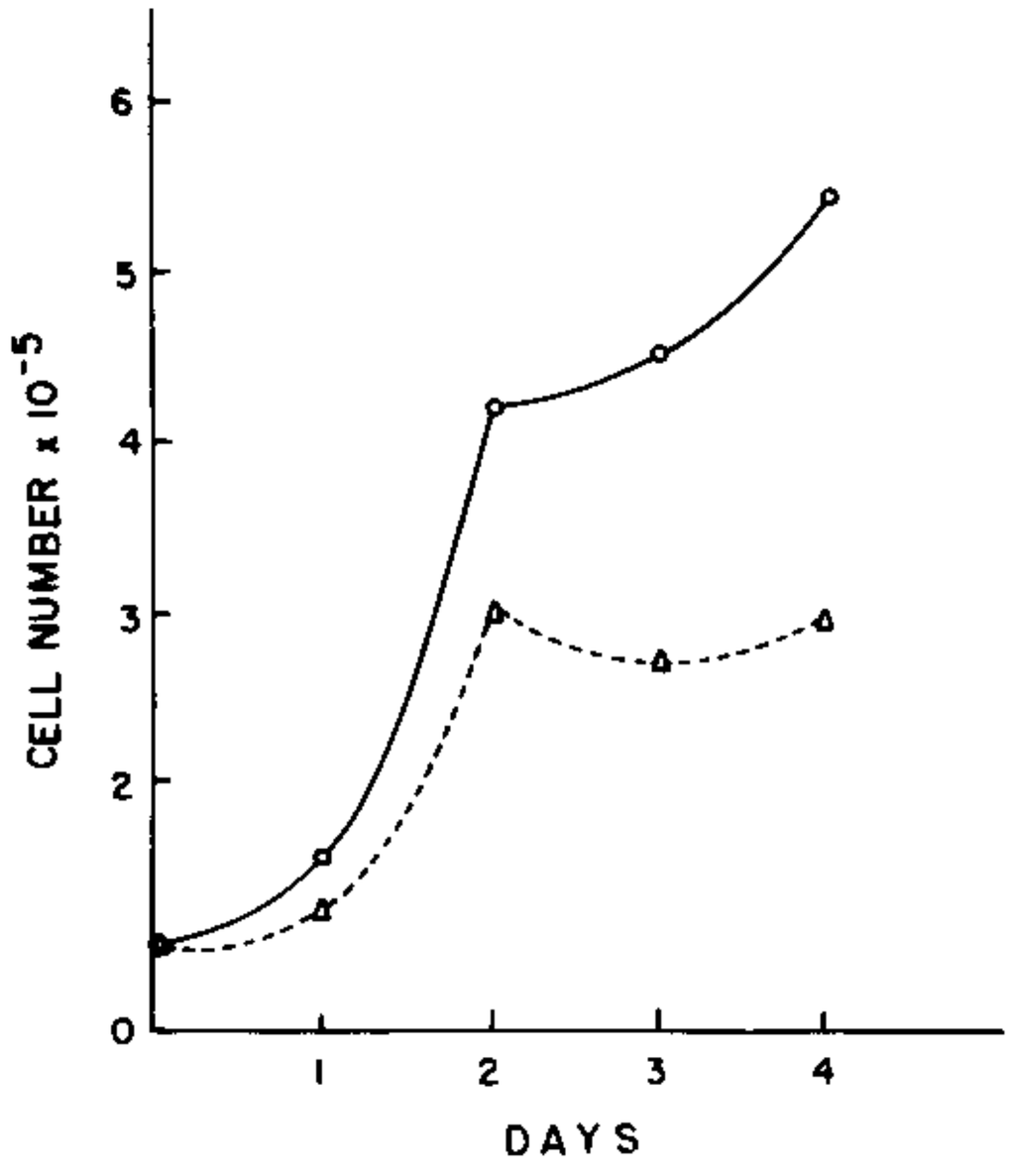

Fig. 1: human lymphoblastoid cells (Daudi) were grown in the presence of human amniotic membrane interferon (80 units/ml, aproximately 10^3 units/mg of protein (- - △ - - △ - -) or without interferon (– ○ – ○ –). Cell counts were done using a hemacytometer.

Anticellular activity – Daudi cells were grown in the presence of 80 units of concentrated IFN-AM (approximately 10 units IFN/mg of protein) and cell numbers were counted every day with the help of an hemacytometer. As shown in Fig. 1, from the second day on, the numbers of Daudi cells treated with IFN showed a significant decrease when compared to controls.

Several dilutions of concentrated IFN (specific activity-about 10^3 units/mg of protein) and IFN purified on a CPG column (specific activity 2 x 10^5) were added to cultures of Daudi cells and the antiproliferative effect determined by cell counts on the third day. Both preparations (Fig. 2) were able to diminish Daudi cell growth. However, the purified preparation required IFN concentrations more than 10 times higher than the concentrated preparation for a 50% inhibition.

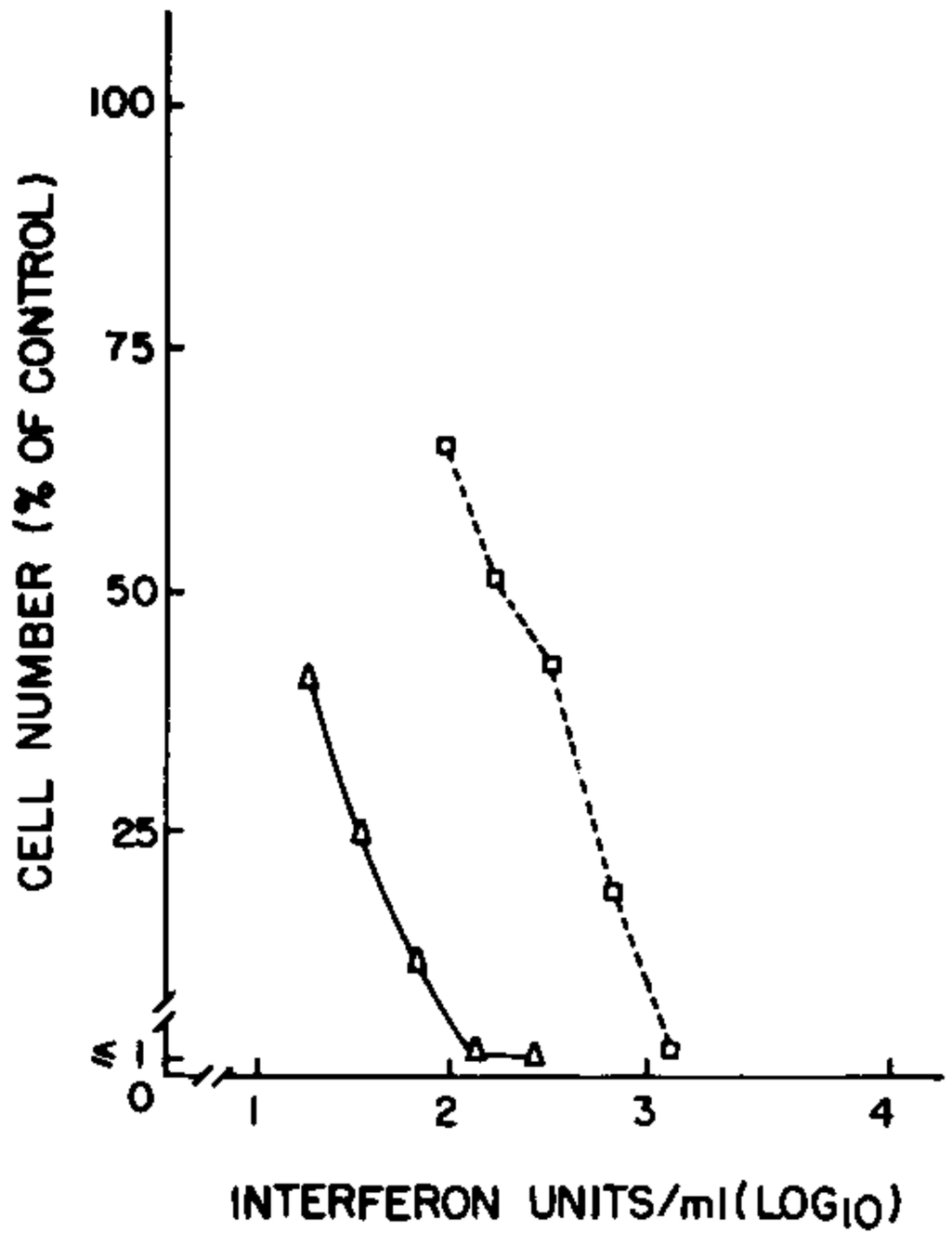

Fig. 2: human lymphoblastoid cells (Daudi) were grown in the presence of concentrated (– △ – △ –) or purified (- - - □ - - □ - -) human amniotic membrane interferon or medium (cell control). On the third day, cell counts were performed using a hemacytometer and the percent of control cell number calculated:

$$\frac{\text{treated cell number (3rd day) - cell number (1st day)}}{\text{control cell number (3rd day) - cell number (1st day)}} \times 100$$

The antiproliferative effect of IFN-AM was then related to other human IFNs. When the numbers of Daudi cells were counted on the third day of treatment (Fig. 3), they demonstrated that all human IFNs caused an inhibition of Daudi cell multiplication. The data suggest that IFN-AM was not as active as alpha and gamma IFNs.

NK cell activity stimulation – Total mononuclear cells from four donors were incubated

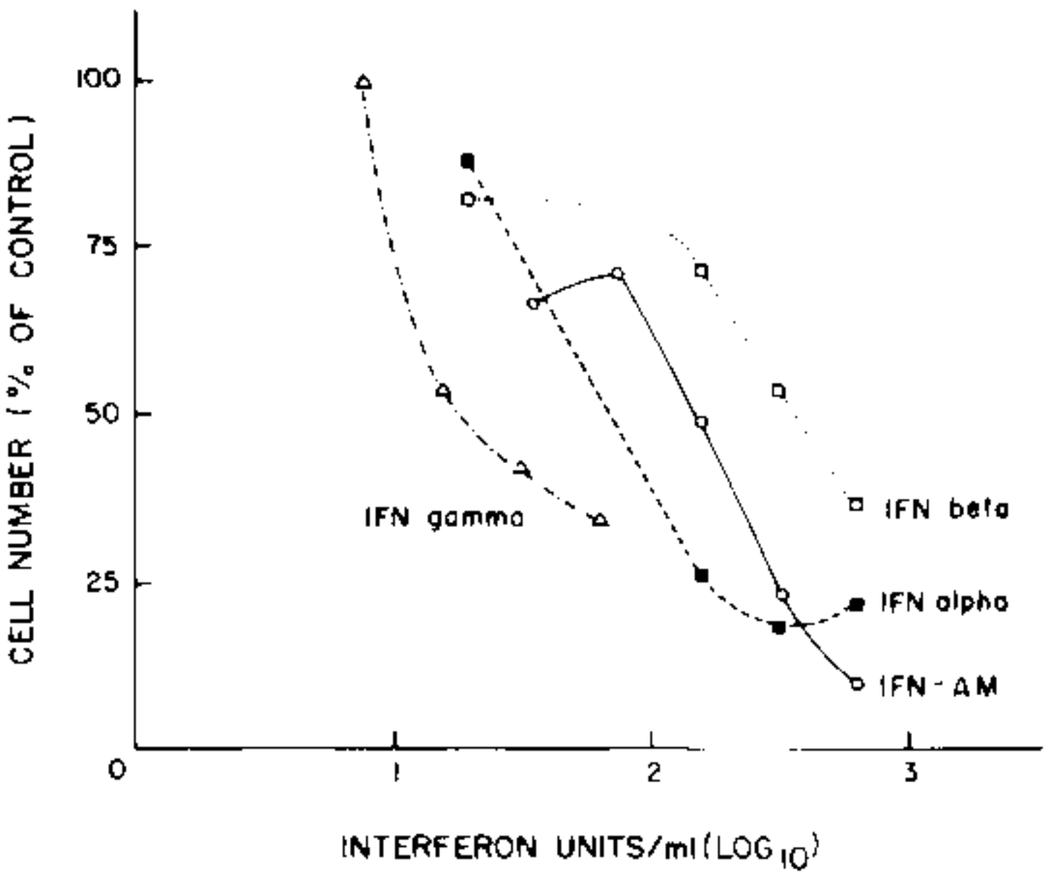

Fig. 3: human lymphoblastoid cells (Daudi) were multiplied in the presence of different concentrations of human interferons. Cell counts were performed and % cell control calculated as in Fig. 2. Specific activities: alpha interferon (IFN) – 7 x 10^4 units/mg of protein; beta IFN – 2 x 10^4; gamma IFN – 10^2; IFN – AM (amniotic membrane) 2 x 10^5.

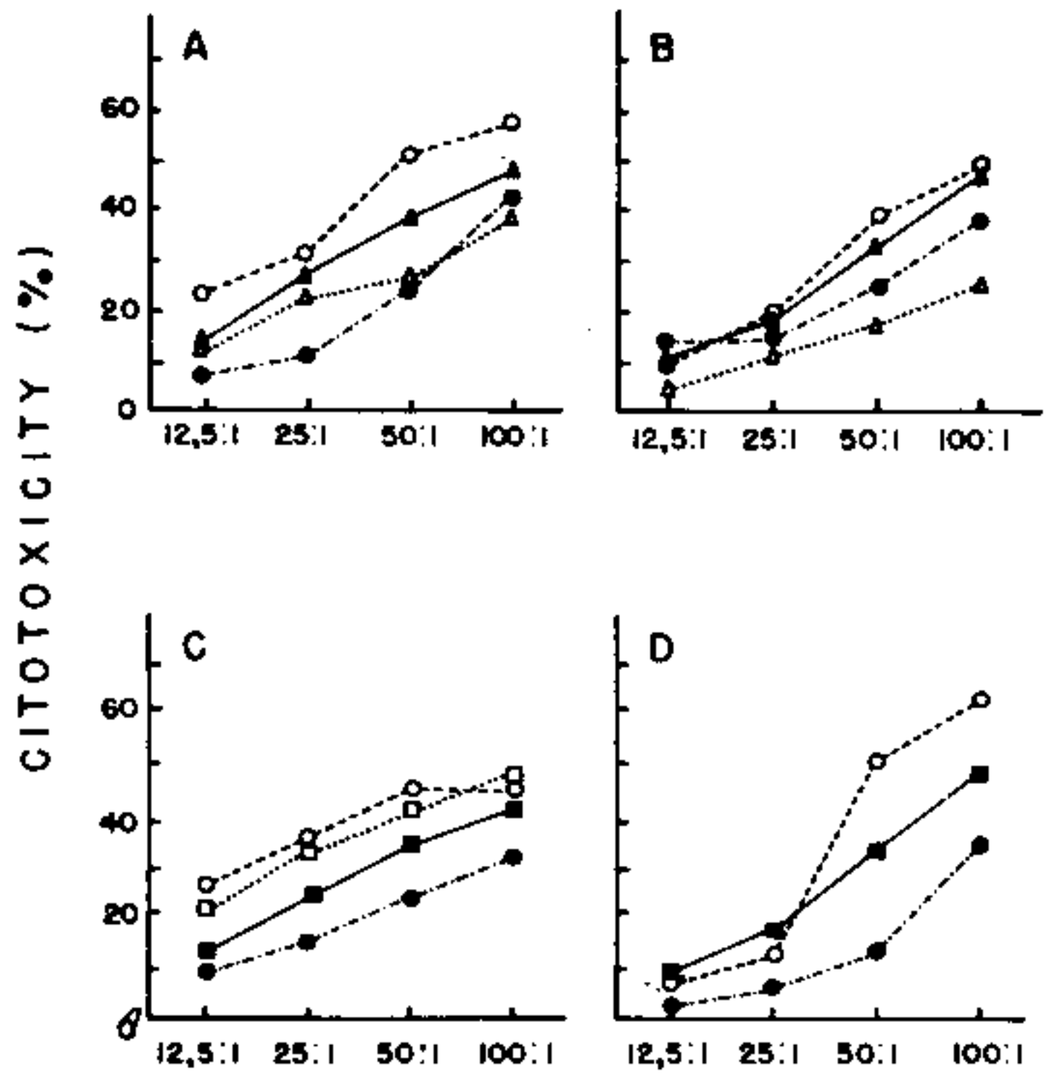

Fig. 4: effect of human amniotic membrane interferon on NK cell activity of effector cells from donors A, B, C and D. Different IFN concentrations were used 20 units/ml (.... △ .... △ ....); 50 units/ml (– ■ – ■ –); 200 units/ml (– ▲ – ▲ –); 250 units/ml (.... □ .... □ ....); 1,000 units/ml (– – – ○ – – – ○ – – –); no IFN (. – . – ● – . – ● – . –). Target cells used were K 562 cells.

separately for 1 h in the presence of various concentrations of IFN-AM (specific activity – 3 x 10^5 units IFN/mg protein). The NK activity was then assayed as described. The results are organized in Fig. 4. Maximum stimulatory effect was obtained with 1,000 units/ml. No activity was seen with 20 units/ml. With 50 units/ml the stimulation was marginal. This effect was general and constant, since in 29 assays using effector cells of 10 donors repeatedly (from 1 to 6 times), and a concentration of 1,000 units/ml of IFN-AM, 24 assays showed statistically significant rise in NK cell activity (data not shown).

The stimulation of the NK activity by IFN-AM was compared with that of other human IFNs, namely alpha and beta, using 1,000 units/ml for all IFNs and effector cells of four donors (Table II). IFN-AM consistently displayed higher NK activity than other human IFNs with the two effector: target cell ratios used. When the NK activity with IFN-AM and alpha IFN was determined using 50 units/ml, similar data were obtained (not shown).

TABLE II

Comparison of the stimulatory effect on NK activity by various human interferons (1,000 U/ml)

Cell Donor	Effector-target[a] Cell Ratio	Control	Percent Cytotoxicity IFN[b] Amniotic	Alpha	Beta
1	50:1	32	47	43	18
	25:1	19	37	28	12
2	50:1	4	11	6	6
	25:1	1	5	2	3
3	50:1	24	33	18	ND[c]
	15:1	13	18	11	ND
4	50:1	18	39	27	ND
	25:1	11	23	17	ND
	50:1	36	44	36	ND
	25:1	28	33	28	ND

a: effector cells: total mononuclear; target cells: K 562.
b: specific activities: amniotic membrane IFN – 3 x 10^5 U IFN/mg of protein; recombinant alpha 2 IFN-greater than 2 x 10^8; beta IFN-10^5.
c: not done.

Control experiments where target cells were incubated directly with IFN-AM did not show cytotoxicity. Also, if the effector cell population was depleted of macrophages, no evidence of a significant decrease in NK stimulation of IFN-AM was seen. When untreated total mononuclear cells at a ratio of 50:1 were used, a median of 20% cytotoxicity was seen, whereas macrophage depleted effectors produced 25%. IFN treatment elevated the percentage of total effector cells to 31%, whereas macrophage depletion resulted in 39% cytotoxicity.

DISCUSSION

In general, IFNs show "species specificity" in antiviral action, that is, they only protect cells from homologous and phylogenetically related species. However, human alpha IFN is able to induce an antiviral state in bovine cells, whereas human beta or gamma IFNs can not exert this effect (Stewart, 1981). The degree of cross-protection in RV-IAL cells was slightly higher with IFN-AM when compared to the activity of beta or gamma IFN, but significantly less than that of alpha IFN (Table I). This might be due to a different sensitivity of RV-IAL cells as related to the cell lines used in other reports or that IFN-AM is a mixture of IFN molecules that differ in their "species specificity". The data of Duc-Goiran et al. (1983) and Reis et al. (1989) support this last hypothesis.

IFN-AM has anticellular activity (Fig. 1) as shown by other human IFNs. The inhibition of Daudi cells growth could be seen clearly on the second day and reached a peak after three to four days of treatment (Fig. 1); a similar situation was described for human beta (Kataoka et al., 1982) and alpha IFN (Evinger et al., 1981) with the same cells. Pure IFNs have antiproliferative effects, but preparations may also contain impurities that hamper cell growth (Golgher & Paucker, 1970; Dahl & Degre, 1975). The results (Fig. 2) showed that 16 units/ml of concentrated and 320 units/ml of purified IFN-AM were needed to inhibit Daudi cell growth to 57%, indicating that the concentrated preparations of IFN-AM had impurities that participated in the anticellular activity of IFN-AM. However, since purer preparations (in excess of 10^6 units per mg of protein) also displayed similar activity (data not shown), it can be concluded that IFN-AM, by itself, can inhibit cell growth. The curves presented in Fig. 3 suggest a close relationship between human beta IFN and IFN-AM. These data, taken together with the physicochemical characteristics of IFN-AM as compared to other human IFNs (Ferreira et al., 1991) and the antigenic properties of IFN-AM (Reis et al., 1989), clearly indicate that this IFN contains a mixture of a major component similar but not identical to human beta IFN and other component(s) yet to be investigated.

Interferons have a marked stimulation on NK cell activity *in vitro* (Herberman, 1984) but types of human IFN differ in their capacity to exert this effect (Ortaldo et al., 1982; Okabe et al., 1985). IFN-AM, which had never before been tested in relation to NK activity, was shown also to augment this cytotoxicity in a dose-dependent manner (Fig. 4). That this effect was not due to direct toxicity of the IFN-AM on the target cell, or to toxicity of its impurities, was demonstrated by the fact that incubation of this cytokine with target cells did not lead to cell death. The response to this enhancer, however, can vary according to the donor and daily fluctuation in sensitivity of target cells. This variability has also been described for other IFNs (Goldfarb & Herberman, 1981; Uchida et al., 1984). The rise in cytotoxicity induced by IFN-AM was not dependent on basal NK activity of the individuals studied, that is, this augmentation was seen whether the original cytotoxicity was high or low (data not shown). That IFN-AM did not exert its stimulatory effect on NK activity through the aid of macrophages was seen by the fact that the removal of these cells did not alter the pattern of response, in accordance with what has been reported for other IFNs (Platsoucas et al., 1986). Our data (Table II) suggest that IFN-AM has a higher NK stimulatory activity than human IFNs alpha and beta *in vitro*. However, further experiments with pure IFN-AM are needed to confirm this finding.

Because of its anti-cellular and NK stimulatory activity, IFN-AM is indicated as a potential antitumor drug.

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