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HUMAN LEUKOCYTE INTERFERON SUBTYPES HAVE DIFFERENT ANTIPROLIFERATIVE AND ANTIVIRAL ACTIVITIES ON HUMAN CELLS

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The antigrowth effects of 5 different cloned human leukocyte IFN subtypes (IFN-αA, B, C, D, F) and 2 molecular hybrids between them (IFN-αAD(BglII) and IFN-αDA(BglII)) were examined on 6 different human cell lines. The results indicate that the interferons sort into two distinct groups: IFN-αB, C and F showed comparable antiproliferative activity which was greater than that of IFN-αA, D, AD(BglII) and DA(BglII). The interferons could also be assigned to one of two groups on the basis of their antiviral activity. IFN-αA, D and AD(BglII) were observed to be more protective than IFN-αB, C and F against HSV-2 and EMCV infections, i.e. the relative antiviral efficacies of the cloned IFN subtypes were the reverse of their antiproliferative activities.

Cell growth inhibitory activity has been observed with mouse interferon preparations ranging from crude tissue culture-derived extracts to purified IFN preparations (1-10) and with human interferon preparations (11-18) both in vitro and in vivo. Variations in cell growth inhibitory effects are seen with different IFN preparations as well as with the different target cells employed. Certainly differing degrees of inhibition are not uncommon when normal, hyperplastic and

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The abbreviations used are: IFN-α, human leukocyte interferon with the amino acid sequence of sub-types -A, -B, etc., as defined elsewhere (19,21); U/ml, international standard units/ml; WISH, human amnion cells; MDBK, bovine kidney cells; HSV-2, herpes simplex virus type 2; EMCV, encephalomyocarditis virus.

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neoplastic tissue of the same histologic origin are examined and the relation between cell growth inhibitory activity and antiviral activity remains unclear.

Cloning of genes for IFNs in bacteria has revealed the existence of a family of human leukocyte IFN subtypes (HuIFN-α) (19). The various subtypes show distinct antiviral activites with different potency against several viruses in a range of mammalian cell lines (20) and the hybrid IFN IFN-αAD shows even greater antiviral activity on mouse cells than on human cells (21). Some antiproliferative activity against human cells has been demonstrated for two subtypes, IFN-αA and D (22-24)and for the hybrid, IFN-αAD, against mouse cells (25,24). However, to date purified subtypes have generally shown antiproliferative activity lower than that of buffy coat IFN preparations, which are mixtures of the various subtypes (19,24,26).

This study reports that some HuIFN-α subtypes exert a considerable degree of growth inhibitory activity in human cells. In an attempt to determine whether the growth inhibitory activities of the respective IFN subtypes correlated with their antiviral activities, preliminary studies were undertaken using 2 of the same cell types challenged with herpes-virus hominis type 2 (HSV-2) and encephalomyocarditis virus (EMCV). In order to compare a sufficient number of different HuIFN-α subtypes, some of the preparations used were impure. However, various experiments indicate that the differences in the properties observed are not simply due to contaminants.

MATERIALS AND METHODS

Cell cultures

Six different human cell types were used in this study: T98G (neuroblastoma), SK-H-NA (hepatoma, provided by L. Heller from the Memorial-Sloan Kettering Cancer Research Center, New York), PLC-PR-5 (27,28) (hepatoma, expressing hepatitis B surface antigen, provided by B. Shames and S.V. Feinman, Mount Sinai Hospital, Toronto), K562 and HL60 (2 distinct myeloid leukemic lines) and Daudi (a lymphoblastoid line, derived from B-cells). Cells are grown either as monolayer or suspension cultures at 37°C in humidified air with 5% CO_2, in MEM-α medium (University of Toronto tissue culture medium preparation service) supplemented with 10% fetal calf serum (Gibco), 10^3 I.U. penicillin and 10^3 µg/ml streptomycin.

Viruses

HSV-2 stain no. 333, isolated from a primary genital lesion and provided by Dr. Carlos Lopez of the Memorial Sloan-Kettering Cancer Center, New York, was used in this study. HSV-2 is routinely passaged on WI-38 human lung fibroblasts and EMCV on mouse L-929 cells.

Interferons

Five HuIFN-α subtypes obtained by gene cloning in E. coli were used: IFN-αA, B, C, D and F (19). These interferon subtypes all consist of 166 amino acid residues, except IFN-αA, which consists of 165 amino acid residues (19). The hybrid interferons IFN-αAD(BglII) and IFN-αDA(BglII) used consist of the N-terminal 61 or 62 residues of IFN-αA or D respectively and the remaining C-terminal portion of IFN-αD or A, respectively (21). These recombinant IFN-αs were kindly provided by Genentech, Inc., San Francisco, CA. The unpurified IFN-αs B,C and F were prepared as described previously and had specific activities in a range from 3.2×10^4 to 1.6×10^5 U/mg protein (20). Extracts of bacterial cells containing plasmids lacking IFN gene sequences were found to have no antiviral activity. In addition, we used preparations of IFN-αA, D, AD(BglII) and DA(BglII) which were electrophoretically homogeneous and greater than 95% pure with specific activities between 0.6 and 2.2×10^8 U/mg protein, assayed on MDBK cells as described elsewhere (21). A control human buffy coat IFN preparation was obtained from Dr. Norwood Hill, Wadley Institute for Molecular Medicine, Houston, Texas. This natural material had a specific activity of 1×10^6 U/mg protein. In addition, lymphoblastoid IFN was used (Wellcome Foundation, U.K.) with a specific activity of 2×10^8 U/mg protein. IFN titers were determined on WISH or MDBK cells challenged with VSV using a 50% cytopathic end-point and converted to international units using an NIH standard (G023-901-527) included in each assay. Rabbit anti-leukocyte IFN was obtained from Interferon Sciences, Inc. New Brunswick, NJ.

In Vitro Assay for Antiproliferative Activity

Cells were seeded in individual wells of 96-well Microtest II tissue culture plates (Falcon, Oxnard, Ca.) at a density of 10^3 cells/well (5×10^3/ml) for the monolayer cultures PPL-PR-5, SK-H-NA and T98G, and at 2×10^3 cells/well (10^4/ml) for the suspension cultures K562, Daudi and HL60. These are the optimal seeding concentrations to avoid early confluence of cultures. The growth inhibitory activities of the various interferons were determined by inoculating cultures at the times of their initiation with dilutions of standardized doses of the IFNs. At predetermined times, monolayer cells were ethanol fixed, crystal violet (0.1% in 2% ethanol) stained and inhibition of growth estimated from absorbance at 570 nm using a Microplate Reader MR600 and a calibration of absorbance against cell numbers. Suspension culture cell aliquots were counted directly in a Coulter Counter (model ZB1).

The growth inhibitory activity of the various IFN-αs is expressed as a percent of control growth. For the monolayer cultures, the absorbance at 570 nm of cells not treated with IFNs and stained with crystal violet served as a standard for normal growth. For cells fixed and stained during their logarithmic phase of growth, their absorbance at 570 nm is directly proportional to the number of cells present, for all the monolayer cell types used. IFN-treated cells were scored for percent of control growth by comparing their absorbances at 570 nm with corresponding cell standards. Similarly, IFN-treated suspension cultures were scored for percent of control growth by comparing their cell numbers directly with cell standards.

In Vitro Assay for Antiviral Activity

T98G cells were seeded in individual wells of 96-well Microtest II tissue culture plates and pretreated with dilutions of the respective IFN preparations for 24 hrs. At the time of virus inoculation the IFNs were removed and either 3 µl HSV-2, $TCID_{50}$ $10^{-4.0}$ (determined on Vero cells) or 10^4 PFU EMCV added to individual wells in 100 µl medium. To terminate the experiment cells were fixed with 95% ethanol. The extent of HSV-2 and EMCV infection was determined by spectrophotometric estimation of viral cytopathic effect: fixed cells were crystal violet stained and the inhibition of virus infection estimated from absorbance at 570 nm.

Antiviral assays were also set up using K562 cells. Cells were seeded in wells of the Microtest culture plates and pretreated with the respective IFN preparations for 24 hrs. The IFNs were then removed and either HSV-2 or EMCV added as above in 100 µl medium. Viral adsorption was permitted for 2 hrs after which time the medium was replaced with fresh medium. Cells were incubated for 23 hrs then assayed for virus yield by quantitating viral CPE on Vero monkey kidney cells.

RESULTS

Antiproliferative Activities

The growth inhibitory effects of the various IFN-αs were examined on human monolayer and suspension cultures. Figures 1 and 2 show typical experiments in which inhibition of cell growth was measured. The subtypes IFN-αB, IFN-αC and IFN-αF demonstrated pronounced inhibition of cell growth that exceeded the inhibition observed with the subtypes IFN-αA and IFN-αD and the hybrids IFN-αAD(BglII) and IFN-αDA(Bgl). Gross changes in morphology and growth patterns of the different cell types were not observed with IFN treatments. Where growth was completely suppressed cells eventually degenerated. Overall the IFN-αDA (BglII) hybrid demonstrated the least antigrowth activity. The extent of growth inhibition induced by the various IFN preparations for the different cell types is summarized in Table I expressed as concentrations causing 50% inhibition of growth. The IFN-αs B, C and F, at 10^3 U/ml, inhibited the growth of all the cell types except SK-H-NA, by 60-70%. For the SK-H-NA line, these IFNs inhibited growth to a lesser extent, by 2-19%. For the IFN-αs A, D, AD (BglII) and DA (BglII), at 10^3 U/ml, growth was inhibited by 0-25% on K562, HL60, T98G and SK-H-NA cells. The Daudi and PLC-PR-5 lines were more sensitive to certain of these IFNs: At 10^3 U/ml, IFN-αs A, D and AD (BglII) inhibited growth

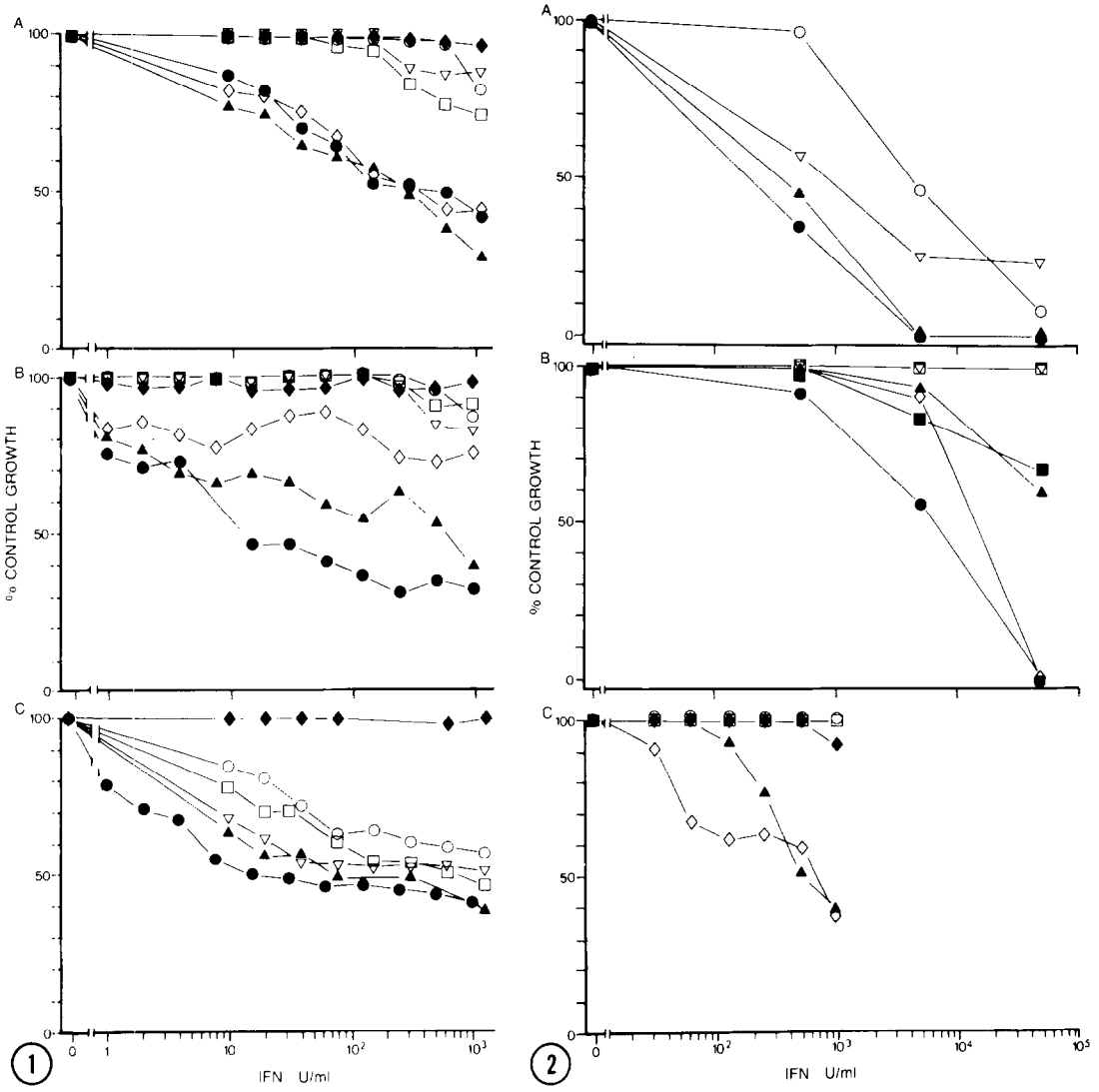

Figure 1 Growth inhibitory activities of HuIFN-αs on human suspension cultures.
A, K562; B, HL60; C, DAUDI;
Cells (10^4/ml) were incubated in MEM-α medium containing 10% fetal calf serum at 37°C in the presence of an IFN for 98 hrs (K562), 118 hrs (HL60) and 96 hrs (DAUDI). Cell numbers were counted in a Coulter Counter. The values are the average of replicate samples each.
IFN-αA (□); IFN-αB (●); IFN-αC (▲); IFN-αD (○);
IFN-αF (◇); IFN-αAD(BglII) (▽); IFN-αDA(BglII) (◆).

Figure 2 Growth inhibitory activities of HuIFN-αs on human monolayer cultures.
A, PLC-PR-5; B, SK-H-NA; C, T98G;
Cells ($5x10^3$/ml) were incubated in MEM-α medium containing 10% fetal calf serum at 37°C in the presence of an IFN for 144 hrs (PLC-PR-5), 125 hrs (SK-H-NA) and 112 hrs (T98G). Cell numbers were estimated by spectrophotometric determination. The values are the average of replicate samples each.
IFN-αA (□); IFN-αB (●); IFN-αC (▲); IFN-αD (○);
IFN-αF (◇); IFN-αAD(BglII) (▽); IFN-αDA(BglII) (◆);
buffy coat IFN (■).

of Daudi cells by 42-52%. Similarly, at 10^3 U/ml, AD (BglII) inhibited growth of PLC-PR-5 cells by 52%. Overall these results indicate that whereas Daudi cells are most sensitive to the growth inhibitory effects of the range of IFN-αs tested, the SK-H-NA cells are least sensitive.

To test whether impurities in the crude preparations IFN-αB, -αC and -αF may influence the growth inhibitory activities of these IFNs the antiproliferative activity of IFN-αC was determined on mouse L-cells. Any bacterial extract impurities present in the crude IFN-αC preparation that might inhibit growth of human cells should likewise inhibit the growth of mouse cells. Whereas a pure preparation of IFN-αAD(BglII) at 20 and 500 U/ml inhibited the growth of L-cells by approximately 30 and 65% respectively, IFN-αC at both concentrations had no inhibitory effect on the growth of L-cells. Furthermore, extracts of bacterial cells containing plasmids lacking interferon gene sequences were found to have no growth inhibitory activity, and a human leukocyte interferon specific antibody preparation completely abrogated the anti-proliferative effects of IFN-αC.

Antiviral Activities

The antiviral effects of the interferon subtypes were examined on T98G and K562 cells challenged with HSV-2 and EMCV. The data, shown in

TABLE I

GROWTH INHIBITORY ACTIVITIES OF HuIFN-αs ON DIFFERENT HUMAN CELL TYPES

CELL TYPE	ID_{50}[a] IFN-αA	IFN-αB	IFN-αC	IFN-αD	IFN-αF	IFN-αAD (Bgl II)	IFN-αDA (Bgl II)	BUFFY COAT IFN	LYMPHOBLASTOID IFN
T98G	$>10^3$	-	5.2×10^2	$>10^3$	6.7×10^2	-	$>10^3$	-	-
PLC-PR-5	-	2×10^2	3.5×10^2	4.3×10^3	-	8.7×10^2	-	-	-
SK-H-NA	$>5\times10^4$	6.5×10^3	-	-	1.4×10^4	$>5\times10^4$	-	$>5\times10^4$	-
DAUDI	7.2×10^2	15.6	70	$>10^3$	-	$>10^3$	$>10^3$	9×10^2	-
K562	1.25×10^3	6.25×10^2	3×10^2	$>1.25\times10^3$	3.7×10^2	$>1.25\times10^3$	$>1.25\times10^3$	-	$>1.25\times10^3$
HL60	$>10^3$	13	5.9×10^2	$>10^3$	$>10^3$	$>10^3$	$>10^3$	-	$>10^3$

Dilutions of the different IFN subtypes and hybrids were introduced into individual wells at the time of seeding with the appropriate cell type. Replicate assays were set up in all cases and the mean values of cultures assayed between 4-6 days are represented above.

[a] IFN concentration, U/ml, causing 50% growth inhibition

- not done

Figure 2, demonstrate that the relative antiviral activities of the different IFN preparations is distinct from their relative antiproliferative effects in the same cells, i.e. IFN-αA, IFN-αD and IFN-αAD (BglII) are more antiviral than the subtypes IFN-αB, IFN-αC and IFN-αF. Protection was more pronounced with the IFN-αs A, D and AD (BglII) against both virus infections in K562 cells, where viral CPE was reduced by 85-98%. The extent of antiviral activity induced by the various IFN preparations against HSV-2 and EMCV infections of K562 cells is summarized in Table II.

DISCUSSION

The relative growth inhibitory activities of each of the cloned HuIFN-αs remained the same on all the cell types tested. Where the growth rate was severely suppressed the cells eventually died. The inhibitory activities of IFN-αB, C and F were comparable and greater than IFN-αA, D, AD(BglII), DA(BglII) and buffy coat and lymphoblastoid interferon, when similar titers of the interferons were used in the various cell lines. It should be noted that the differences in efficacy between IFN-αDA(BglII) and either of the parental IFN subtypes, IFN-αA and D or the reverse hybrid, IFN-αAD(BglII) supports previous results indicating that neither the N- or C-terminal portions of the

TABLE II

ANTIVIRAL ACTIVITIES OF HuIFN-αs ON K562 CELLS

VIRUS	ID_{50}[a] IFN-αA	IFN-αB	IFN-αC	IFN-αD	IFN-αF	IFN-αAD (Bgl II)	IFN-αDA (Bgl II)
EMCV	1.75×10^2	-	5×10^2	2×10^2	$>10^3$	70	$>10^3$
HSV-2	1.45×10^2	-	$>10^3$	2.5×10^2	$>10^3$	37	$>10^3$

K562 cells were pre-treated for 24 hrs. with dilutions of the different IFN-αs then challenged with either EMCV or HSV-2. After 23 hours virus yield was assayed on Vero cells.

[a] IFN concentration, U/ml, causing 50% CPE on Vero cells

- not done

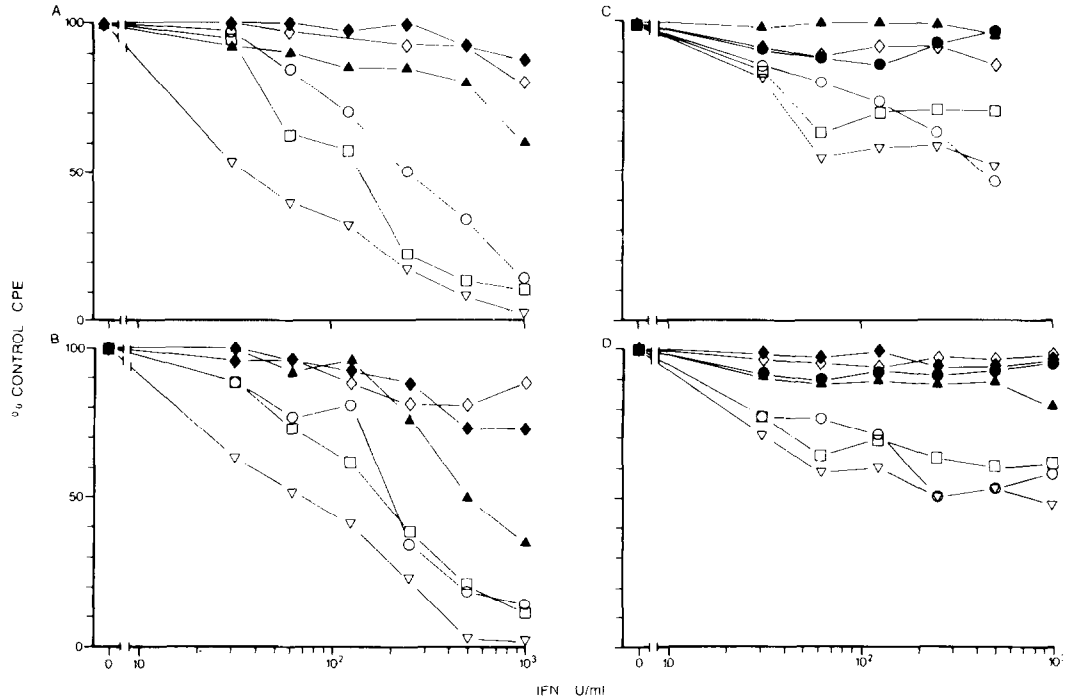

Figure 3 Antiviral activities of HuIFN-αs on human cells.
A, K562 + HSV-2; B, K562 + EMCV; C, T98G + HSV-2; D, T98G + EMCV;
Cells were pre-treated with an IFN for 24 hrs then challenged with either HSV-2 or EMCV. For A and B virus yield was determined by resultant CPE on Vero cells, and for C and D viral CPE was quantitated directly by spectrophotometric estimation. The values are the average of replicate samples each.
IFN-αA (□); IFN-αB (●); IFN-αC (▲); IFN-αD (○);
IFN-αF (◇); IFN-αAD(BglII) (▽); IFN-αDA(BglII) (◆).

interferons alone determine cell receptor binding (29,30).

IFN-αA, D and AD(BglII) were observed to be more effective as antiviral agents against cells challenged with HSV-2 and EMCV than IFN-αB, IFN-αC or IFN-αF. These results suggest that the various leukocyte IFN subtypes used have distinct biological properties that may prove useful for selection as either antigrowth or antiviral agents (Figure 3).

Although not all the studies reported here included use of highly purified IFN preparations, the results do not seem to depend on the degree of purification since a crude bacterial extract had no growth inhibitory activity at all, and an antibody preparation abrogated the growth inhibitory activity of a crude preparation of IFN-αC. The

interferon antibody preparation should not neutralize any of the bacterial contaminants because the antibody was raised in rabbits against human leukocyte IFN.

Whether inhibition of cell multiplication and inhibition of viral replication are mediated by the same mechanisms remains uncertain. Various IFNs might induce different biochemical pathways that might have differential effects on cell growth or virus replication (20,26,31). The observation that those IFNs with the greatest antiproliferative activity did not demonstrate the greatest antiviral activity suggests that there is no relation between the antigrowth and antiviral effects of a particular IFN subtype. This result may have important implications regarding the selection of recombinant-DNA derived IFN subtypes for particular clinical indications. The various cloned leukocyte IFN subtypes have been classified into two major groups and an intermediate group, depending on alternative amino acid residues to be found at defined positions, namely 14, 16, 71, 78, 79, 83, 154 and 160 (32). IFN-αA and D fall into group I whereas IFN-αB, C and F fall into group II, or the intermediate group (32). Thus, the structural differences cited may be the basis for the differences in antiviral and antiproliferative effects observed in the present studies. By extending these studies to include a wider range of cell types and viruses, it should be possible to test this idea further and identify IFN subtypes or hybrids with optimum antiproliferative or antiviral activity. Synthesis of new hybrid interferons from particular parental subtypes could thus produce a new generation of IFNs more potent as antigrowth or antiviral agents than the respective parental subtypes.

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