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Unusual human interferons produced by virus-infected amniotic membranes

(light and heavy human interferon-α and -β species/amniotic membrane/affinity chromatography)

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ABSTRACT **Interferon (IFN) induced in the human amniotic membrane contains at least five different molecular species, as shown by analysis in $NaDodSO_4$/polyacrylamide gels after heating and under reducing conditions. Three of the IFN components reported here—migrating at 26, 43, and 80 kilodaltons—are of unusual antigenic structure because they are neutralized to about the same extent by anti-IFN-α and anti-IFN-β antibodies. The 15- to 17-kilodalton species belongs to the IFN-α group, while the 21- to 22-kilodalton species, the most frequently detected major peak, is IFN-β. In addition to their unusual size and antigenic structure, these IFNs could play a role during embryonic development and in the immune tolerance of the mother with regard to the fetus.**

Interferons (IFNs) seem to play an important role during natural processes of recovery from viral infections (1). It is also likely that the IFN system contributes to the regulation of a series of cellular events that involve cell growth (2) and the phenotypic expression of normal and malignant cells (3). Furthermore, the great variety of IFN-α species revealed by purification (4, 5) and by genetic engineering (6) suggests that, in addition to common antiviral properties, some of these IFN species could have varied physiological functions. For example, some IFN-α or -β species could be involved in immune regulation or surveillance (7).

An interesting recent development is the demonstration of the involvement of IFN in cell differentiation (8) and the regulation of cytoskeleton (9, 10) and extracellular matrix (11) synthesis. Some of the IFNs could play a role during embryonic development. This hypothesis is supported by the detection of small but significant amounts of IFN-α species in amniotic fluid samples collected from women between the 16th and 38th wk of pregnancy (12, 13). Similarly, IFN was found in pools of apparently uninfected mouse embryos (14).

In the present paper, we report that the IFN released from virus-infected human amniotic membrane is heterogeneous in composition, containing a number of molecular species.

MATERIALS AND METHODS

Cells and Viruses. Human diploid F7000 (Flow Laboratories) and bovine kidney (MDBK) cells were grown in Eagle's minimal essential medium (ME medium)/10% heat-inactivated fetal calf serum. Sendai virus (E72 strain) and vesicular stomatitis virus are routinely maintained in our laboratory.

IFN Production. IFN was produced in human amniotic membranes as described (15). The conditions of induction were modified recently: briefly, the membranes were incubated at 37°C for 24 hr with ME medium/10% heat-inactivated newborn calf serum. Then, the membrane was cut into about 5-g fragments (containing approximately 6×10^6 cells per g of wet tissue) and primed with 10 units of amniotic IFN for 2 hr, infected with Sendai virus at a multiplicity of infection of 1,000 plaque-forming units per cell, and incubated with ME medium/1 mM theophylline at 37°C for 24 hr. Virus was inactivated by the usual acid treatment for 48 hr. IFN activity was assayed by microtitration with vesicular stomatitis virus as challenge on F7000 and MDBK cells. The titer of the crude IFN preparation was usually 10,000–20,000 units/ml and is expressed in international IFN-α reference units (IU; NIH G025 901 527).

Concentration and Purification Procedures. After acid treatment, the first step in our purification scheme was the selective precipitation of crude human amniotic membrane IFN by air-dried ammonium sulfate to remove the bulk of the protein and concentrate the IFN. The precipitate that formed at 0–25% saturation was removed by centrifugation and discarded. The supernatant was then treated with ammonium sulfate at 70% saturation, and the precipitate that formed was collected by centrifugation and dissolved in 0.05 M phosphate buffer. The IFN solution was concentrated to $^1/_{10}$th vol and dialyzed against the phosphate buffer; this fraction contained 150% of the recoverable IFN activity. The specific activity of the crude human amniotic membrane IFN preparations on F7000 cells was $2–6 \times 10^3$ IU/mg of protein; that of the partially purified fractions was $2–7 \times 10^4$ IU/mg of protein.

Hydrophobic Chromatography. CH-Sepharose 4B (ω-carboxypentylagarose) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Ethylene glycol was obtained from E. Merck (Darmstadt, Germany).

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Polyacrylamide gel (15%) electrophoresis in the presence of $NaDodSO_4$ was carried out according to Laemmli (16) and King and Laemmli (17), using vertical slab gels. Crude and purified IFNs and protein markers were dissolved in 200 μl of 0.01 M phosphate buffer (pH 7.2) and mixed with 200 μl of sample buffer (0.1 M Tris·HCl, pH 6.8/2–4% $NaDodSO_4$/10% glycerol/0.6 M bromophenol blue) with or without 9.0 M urea/2% 2-mercaptoethanol. The samples were then heated to 100°C for 1 to 2 min, and 50-μl aliquots were loaded onto the gel.

The polyacrylamide gel was divided into two parts. One part was stained, destained, and dried, and the protein bands were located. The second part was cut into tracks that were then sliced into 3-mm pieces. Each piece was eluted overnight at 4°C for IFN assay. In some experiments, the gel was stained by the periodic acid/Schiff reagent procedure (18) to detect the presence or absence of sugar residues.

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Abbreviations: IFN, interferon; ME medium, Eagle's minimal essential medium; IU, international IFN-α reference unit(s); CH-Sepharose, ω-carboxypentylagarose; kDa, kilodalton(s).

IFN Neutralization Procedure. Various crude and gel-purified IFN preparations were studied for the antigenic structure of their components using antibodies raised against highly purified IFN-α and IFN-β species (the latter was a gift of J. Vilcek). The assays were performed by checkerboard titrations, using simultaneous 1:2 dilution steps for IFN and antibody. The antibody titer was estimated as the concentration of antiserum that reduced 10 IFN IU to 1 (19).

RESULTS

Crude human amniotic membrane IFN preparations have biological and antigenic properties not found in cells producing either IFN-α (leukocyte) or IFN-β (fibroblast). For instance, human amniotic membrane IFN can protect rat embryonic fibroblasts to a relatively high titer (20). This property is not observed with either IFN-α or IFN-β preparations. Furthermore, antibodies raised to both IFN-α and IFN-β neutralize human amniotic membrane IFN to the same extent. It was supposed, therefore, that the crude preparation contained molecular components not detected in other IFN preparations. To explore this possibility, we first applied crude or ammonium sulfate-precipitated human amniotic membrane IFN preparations to CH-Sepharose, which is known to separate IFN-α from IFN-β (21).

Chromatography of Human Amniotic Membrane IFN on CH-Sepharose. The chromatographic behavior of human amniotic membrane IFN was studied on an immobilized low-molecular-weight hydrophobic ligand, CH-Sepharose. The bulk of the proteins that did not bind to CH-Sepharose contained a major IFN peak effective in both human fibroblasts and bovine cells. As shown in Fig. 1, the low-ionic-strength elution step (E1) produced IFN that was active in human fibroblasts and slightly effective in bovine cells. The higher ionic strength (1 M NaCl) elution step (E2) gave IFN that was active in F7000 cells. The proteins tightly bound to CH-Sepharose were eluted only with ethylene glycol (E3). The IFN found in these fractions was active only in human cells.

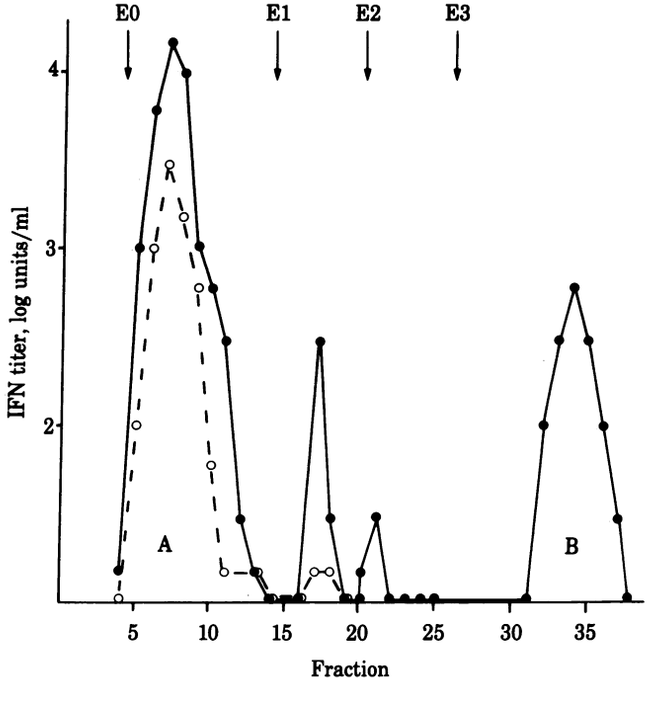

FIG. 1. Chromatography of human amniotic membrane IFN on CH-Sepharose. Ammonium sulfate-precipitated preparations were dialyzed against 0.02 M sodium phosphate buffer (pH 7.4) for 18 hr at 37°C. IFN samples (5 ml) were applied to a column (0.9 × 30 cm), and the column was washed sequentially with 0.02 M sodium phosphate (pH 7.4) (step E0), 0.02 M sodium phosphate, pH 7.4/0.15 M NaCl (step E1), 0.02 M sodium phosphate, pH 7.4/1 M NaCl (step E2), and 0.02 M sodium phosphate, pH 7.4/1 M NaCl/8.5 M ethylene glycol (step E3). Three-milliliter fractions were collected and titrated on MDBK (○) and F7000 (●) cells. Fractions 6–8 and 32–35 were then further analyzed (Fig. 2).

Electrophoretic Characterization of Human Amniotic Membrane IFN. The two major IFN peaks found by chromatography on CH-Sepharose were separately subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis. The antiviral activity was assayed in human fibroblast F7000 and in MDBK cells. Analysis of the first peak (Fig. 2*A*) shows the presence of three major IFN fractions: one migrating in the 80-kilodalton (kDa) zone, active mainly in human but also in bovine cells; another migrating in the 43-kDa area; and a third at 21 to 22 kDa. When assayed in bovine cells, an additional shoulder of activity was detected in the 15- to 17-kDa area. When the tightly bound polyethylene glycol-eluted fraction was analyzed (Fig. 2*B*), a single peak in the 21- to 22-kDa area was found.

For further identification, 16 different ammonium sulfate-concentrated IFN preparations were tested and analyzed by 30 electrophoresis runs, under reducing conditions or not. In each one, for reference purposes, human leukocyte IFN-α was ap-

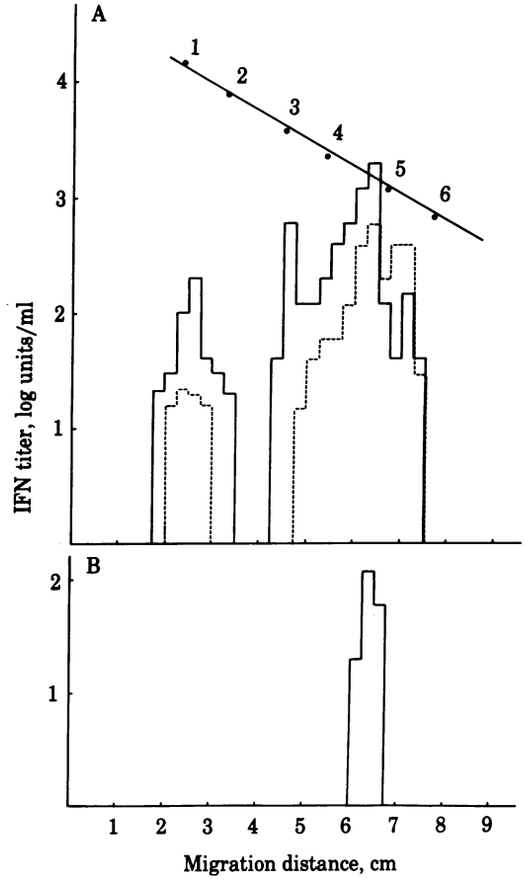

FIG. 2. NaDodSO$_4$/polyacrylamide gel electrophoresis analysis of human amniotic membrane IFN components previously separated by CH-Sepharose. Fractions 6–8 (*A*) and 32–35 (*B*) from Fig. 1 were pooled, dialyzed against 0.03 M ammonium bicarbonate, concentrated by lyophilization, and applied to the gel. Size standards (Pharmacia) were 1, phosphorylase b (94 kDa); 2, bovine serum albumin (67 kDa); 3, ovalbumin (43 kDa); 4, carbonic anhydrase (30 kDa); 5, soybean trypsin inhibitor (20.1 kDa); 6, α-lactalbumin (14.4 kDa). Antiviral activity was measured in F7000 (——) and MDBK (----) cells.

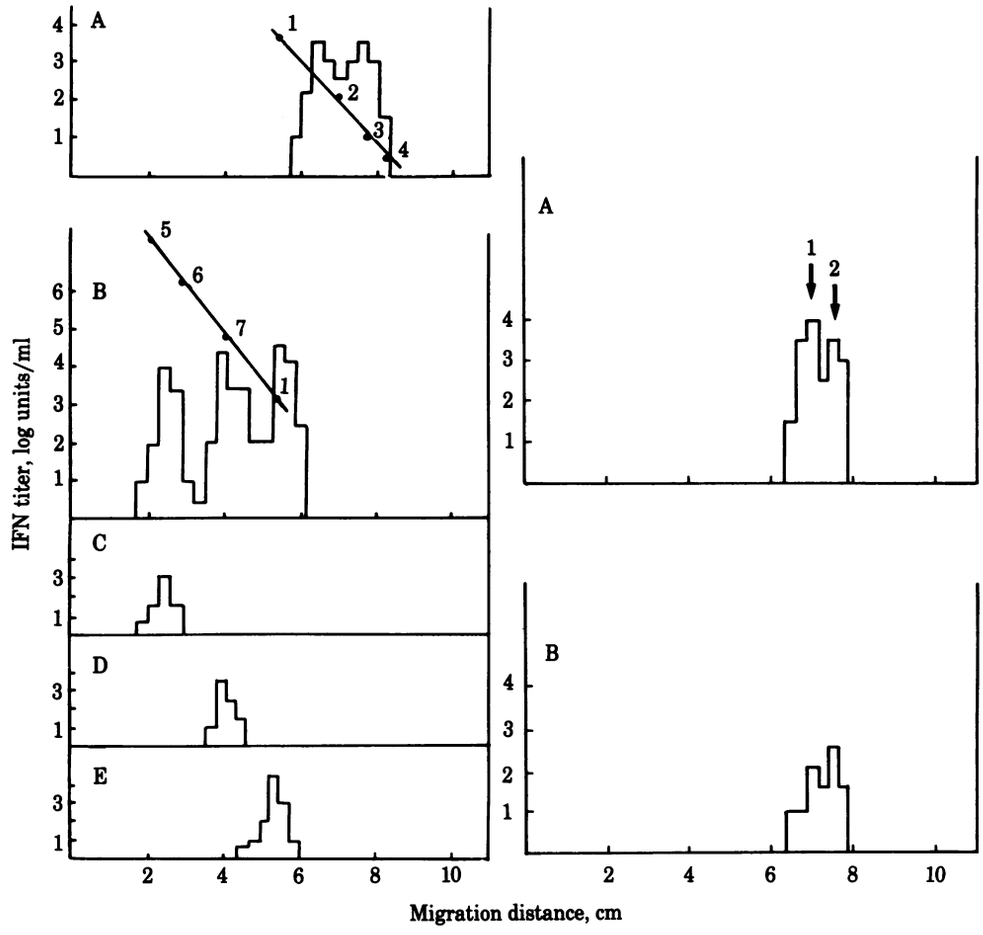

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis analysis of two representative human IFNs. (*Left*) Electrophoretic profiles of reduced control human leukocyte IFN (*A*) and partially purified human amnion IFN (*B*). IFN antiviral effect was assayed in MDBK cells. Size markers were 1, α-chymotrypsinogen (25.7 kDa); 2, β-lactoglobulin (18.4 kDa); 3, α-lactalbumin (14.4 kDa); 4, cytochrome *c* (12.3 kDa); 5, phosphorylase B (94 kDa); 6, bovine serum albumin (67 kDa); 7, ovalbumin (43 kDa). The peak fractions from *B* were then pooled, concentrated, and rerun separately on polyacrylamide slab gels: *C*, 80 kDa; *D*, 43 kDa; *E*, 26 kDa. Their migration patterns were comparable with those in *B*. (*Right*) Electrophoretic profiles of antiviral activity obtained from control human leukocyte IFN (*A*) and partially purified human amnion IFN (*B*) in the lower molecular mass zone. ↓: 1, 21 kDa; 2, 15 kDa.

plied in a parallel track. The three largest forms (80, 43, and 26 kDa) were eluted from the gel, loaded separately on another gel, rerun, and found in the original position (Fig. 3 *Left*). The 21- to 22-kDa and 15- to 17-kDa components, which are the most frequently found, were separately tested (Fig. 3 *Right*). It is of importance that these different molecular species were not detected simultaneously in all experiments; some of them appeared in one and not in another.

Table 1. Frequency and antigenic structure of human amnion IFN species

Protein migration, kDa	Presence, no. of preparations	Antiserum titer* α	Antiserum titer* β	Antigenic structure
15–17	7	6,000	40	α
21–22	16	200	800	β
26	5	1,000	1,600	α, β
43	6	2,000	2,000	α, β
80	7	4,000	1,600	α, β
Crude	16	1,000	1,600	α, β

Sixteen preparations were assayed.
* Expressed as concentration of antiserum that decreases the IFN titer from 10 IU to 1 IU.

Antigenic Identification of Human Amniotic Membrane IFN Components. To characterize the different molecular species found after $NaDodSO_4$ gel separation, each peak was identified by antiserum raised to IFN-α or IFN-β (Table 1). The crude preparation was neutralized to almost the same extent by both IFN-α and IFN-β antisera. The 15- to 17-kDa preparations behaved as IFN-α species; the 21- to 22-kDa can be considered as belonging to the β variant. The 80-, 43-, and 26-kDa components were neutralized by both IFN-α and IFN-β antisera to almost comparable titers. Thus, they seem to share structural components of both species. In summary, amniotic IFN has a complex heterogeneous molecular composition.

To determine whether or not human amniotic membrane IFN preparations contained detectable amounts of carbohydrates, the gel was stained by the periodic acid/Schiff procedure (18) and, in parallel, with Coomassie blue for protein detection. The 80-, 43-, and 21- to 22-kDa fractions seemed to contain sugar residues, while the 26- and 15- to 17-kDa fractions were apparently nonglycosylated (data not shown).

DISCUSSION

The data here reported show that there exists a considerable heterogeneity in the IFNs produced in the amniotic membrane. An earlier report (15) on the presence of high-molecular-

weight components (based on gel filtration) probably represents an overestimation due to aggregation of the molecules or complexes formed with a possible carrier protein. More recent analysis by Ferreira *et al.* (22) also suggests that IFNs of different chemical structures can be found in this population. Their study is based on the interaction of amniotic IFN with various affinity ligands.

The antigenic properties of the crude suspension are also somewhat unusual since antibodies raised to IFN-α and IFN-β neutralize the preparations to almost the same extent. Furthermore, high antiviral activity can be obtained in rat embryonic fibroblasts (20), which is not usually observed with standard IFN-α or IFN-β preparations.

Analysis of the molecular composition of these crude preparations has enabled us to find a reasonable explanation for these discrepancies. Indeed, under reducing conditions, unusually large (43 and 80 kDa) IFN species are found. But in addition to their size, three of the five species also show unusual antigenic structures because these molecules are neutralized to almost the same extent by IFN-α and IFN-β antibodies, indicating that they share antigenic components of both. These species may result from the fusion of originally separated IFN-α and IFN-β species, either at the gene level or during or after transcription. Furthermore, it cannot be excluded that they represent products of currently unrecognized genes; this possibility could be assessed only by recombinant DNA analysis. Moreover, different glycosylation patterns could also explain their unusual migration in $NaDodSO_4$ gels. It is unlikely, however, that these large products result from a post-translational rearrangement of the IFN molecules. This is supported by control experiments in which known IFN-α and IFN-β species were added separately or together to the uninduced amniotic membrane and analyzed on a $NaDodSO_4$ gel (data not shown).

The most frequently detected IFN species, which seems to be predominant, is the 21- to 22-kDa variant identified as IFN-β. The 15- to 17-kDa variant, less frequently seen, is IFN-α. It is difficult to explain the irregular appearance of some of these IFNs from one preparation to another. This could be due to technical reasons. It is likely that the larger components are more unstable than the others. A further possibility could be variation due to the induction procedure. Moreover, some of these IFNs could originate from the placenta (or less likely, the fetus), be absorbed by the amniotic membrane, and released when the inducing virus is applied.

The detection of these different IFNs produced by the amniotic membrane is important in view of the likely role of IFNs in embryonic development. The almost continuous presence of IFN in human amniotic fluid during pregnancy has been reported (12, 13). The levels of IFN are low, but the substance is found in 100% of cases between the sixteenth and thirty-eighth wk of pregnancy. Furthermore, in the mouse, IFN titers increase and peak at the end of gestation (14) while, in the rat, the titers are highest at 15 days and decrease thereafter (unpublished data). It is noteworthy that IFN is not detected in any of the blood samples taken from the mothers.

It is possible that the IFN species described here are not only of unusual size and antigenic structure but also play some yet undefined physiological role. Because they are produced locally, they could be involved in embryonic development or protection of the fetus against maternal immunity. In favor of these hypotheses are the well-documented action of IFN on cytoskeletal structures (9, 10, 23) and its protective effect on T-cell cytotoxicity (24, 25).

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