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Experimental Study of Transplacental Passage of Alpha Interferon by Two Assay Techniques

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Two methods of assaying alpha interferon (IFN-α) were compared during an experiment aimed at determining whether IFN-α crosses the human placenta. Human placentas, collected after delivery following a normal pregnancy to term, were catheterized on both sides: fetal and maternal. The IFN-α was introduced in known amounts in the maternal circulation and was assayed in the efferent fetal fluid. The following two detection methods were used: radioimmunoassay by competition with [^{125}I]IFN-α and assay with a biological system in which IFN-α protected Madin-Darby bovine kidney cells from destruction by vesicular stomatitis virus. The results obtained by the two methods were in perfect agreement for the efferent fetal fluid samples. They showed the absence of placental transfer of IFN-α. The biological method was found to be more sensitive than radioimmunoassay for low IFN-α titers (<10 IU/ml) but was less reproducible, probably owing to the use of twofold dilutions. The specificities of the two methods were similar and their practicalities were equivalent; the biological method, however, was less costly. The study illustrates the complementarity of the two methods, which were based on different principles. The agreement obtained between the two methods provides a clear confirmation of the experimental results.

We report a comparison of two methods for the assay of alpha interferon (IFN-α). Assay of any substance with a specific biological activity can generally be done by several types of techniques. If an antibody (Ab) against the substance is available, it can be used in quantitative immunoassays such as radioimmunologic, immunoenzymologic, and immunofluorometric assays. The activity of the substance can also be measured if investigators have available a suitable biological system which will show a quantifiable modification upon the addition of the substance. The first type of technique is based on recognition of an epitope of the molecule. The second type, whose implementation is often more problematic, is based on the biological activity that develops. In the latter case, it is often difficult to eliminate all interactions between susceptible substances to bring about the same effects (false-positive results) or to inhibit the effect (false-negative results). We opted for a double IFN-α assay: radioimmunology (12) and a biological method described by Lebon et al. (11) that uses the protective effect of IFN-α on Madin-Darby bovine kidney (MDBK) cells, which are sensitive to the vesicular stomatitis virus (VSV) (1, 6).

Transmission of the AIDS virus (human immunodeficiency virus type 1) from mother to fetus is currently a problem of some concern since in Europe the risk is estimated to be 14% (4, 15), and in Africa the risk is estimated to be 40% (8). In order to decrease this type of transmission, treatment with the combination of zidovudine and IFN-α has been considered in pregnant, human immunodeficiency virus type 1-seropositive women (7, 9). Adoption of such a protocol depends on prior knowledge of the possible toxic effects of IFN-α and zidovudine on the developing fetus and the occurrence of IFN-α transport across the placenta.

Zidovudine transport across the placenta has been confirmed in vivo in macaques (14) and ex vivo in humans (2, 13). We therefore studied the transport of IFN-α across the placenta using human placentas (isolated, perfused cotyledon model [16]) in which maternal and fetal vascular systems were supplied with IFN-α ex vivo. IFN-α was assayed by the two methods at the maternal afference (where it was introduced at a known level), the maternal efference, and the fetal efference (i.e., in the liquid from the placenta which, in vivo, would reach the fetus).

MATERIALS AND METHODS

Preparation of placentas. Preparation of placentas was carried out in the laboratory of the Institut National de la Santé et de la Recherche Médicale. It consisted of the collection of a human placenta after delivery following a normal pregnancy to term. A cotyledon of the placenta was chosen. Double perfusion (fetal and maternal) was quickly set up (within the 20 min following delivery). The two perfusions were independent and were performed in an open circuit by the protocol of Schneider and Dancis (16) (see Fig. 1).

The fetal side was perfused by introducing a catheter into a branch of the umbilical artery supplying the placental cotyledon and the corresponding vein. The maternal side was perfused by inserting four cannulae into the intervillous chamber and passing them through the placental tissue; the perfusate was collected by aspiration.

Earle's liquid was used for the perfusion and contained additional plasma proteins (Biotrol 00); it was maintained at 37°C, oxygenated with a mixture of oxygen and carbon dioxide, and pulsed independently by two pumps (one for each circuit). The perfusion was realized when the maternal flow was 13.3 ± 0.7 ml · min^{-1} and the fetal flow was 6.7 ± 0.3 ml · min^{-1}. These values were chosen to reproduce physiological conditions. The flow rates were measured at the inlet and the outlet of the cotyledon (5).

After 25 min of perfusion with the Earle–Biotrol 00 me-

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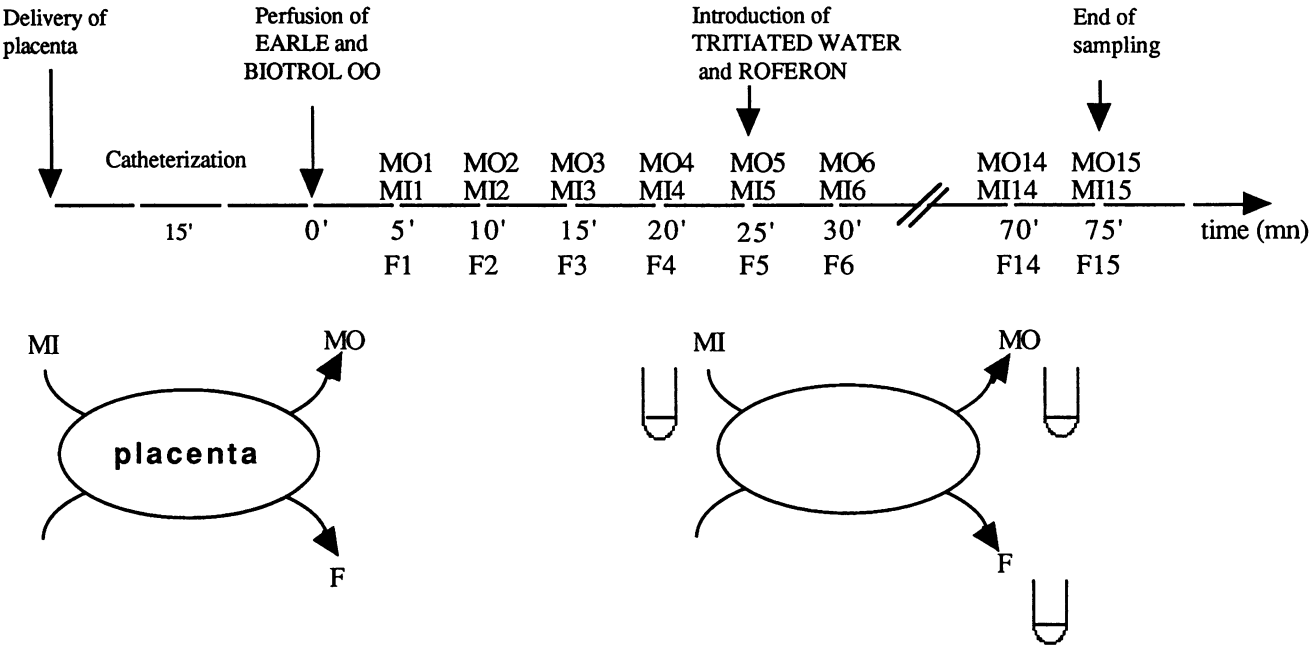

FIG. 1. Preparation of the placentas and sample collection. MI, maternal inlet; MO, maternal outlet; F, fetal outlet.

dium, the molecules to be studied were introduced on the maternal side. A reference substance must not become fixed or degraded by the placental tissue. The transfer rate (TR) was equal to (F/MI) × 100 (where F and MI represent the reference substance concentrations in the fetal outlets and the maternal inlets, respectively) and allowed us to check the integrity of the cotyledon and, therefore, validate the experiment. The TR of the reference substance was compared with that of the studied substance. Antipyrin or tritiated water is generally used as the reference substance (17). In the experiments described here, tritiated water (5 mCi/ml; Amersham France Ltd.) was chosen as a reference marker of placental transfer. It was measured on an LS 1801 Beta Beckman counter (see Table 1).

IFN-α was used at a concentration that was found to be the upper limit of therapeutic doses that can be used in treated subjects (4,000 IU/ml). In the present study we used recombinant human IFN-α_{2a} (Roferon; Roche Laboratories).

The experiments were carried out on four placentas, which is the number regularly used for the kind of study described here. Perfusion lasted for 60 to 75 min. Upon the introduction of IFN-α into the maternal circulation, its concentration reached a steady state after only 15 min. Samples were taken from the fetal and maternal outlets and the maternal inlet every 5 min (Fig. 1). They were immediately centrifuged, decanted, and stored at 4°C until they were assayed (less than 3 h later) and were then frozen at −30°C.

Assays. The samples were assayed in the Virology Laboratory of Purpan Hospital. They were handled rapidly on ice before being frozen at −30°C. Assay of IFN-α in the various samples was carried out by using both radioimmunological and biological techniques:

(i) RIA. Radioimmunoassay (RIA) was performed with the IFN-α Medgenix kit supplied by ERIA Diagnostics Pasteur. It was based on RIA by competition with [^{125}I]IFN-α at a constant concentration plus the IFN-α to be assayed in the samples (18).

The samples or the standards (seven standards with known alpha IFN-α concentrations supplied with the kit) were incubated with a solution of polyclonal rabbit Ab raised against IFN-α. Then, [^{125}I]IFN-α was added (sequential saturation technique) to bind to the Ab sites that remained free.

The Ab–IFN-α complex (bound phase) was separated from the unbound IFN-α (free phase) after precipitation with a mixture of anti-rabbit immunoglobulin G and polyethylene glycol and then centrifugation. The liquid phase was carefully drawn off and discarded. The gamma radioactivity in the tube was then counted for 1 min. This count was used to determine the concentration via the calibration curve set up with the seven standards. The curve, which was obtained after computer processing of the results, showed a linear relationship between the logit of the recorded radioactivity (counts per minute) and the logarithm of the IFN-α concentration. Note that the range over which the calibration curve was plotted was not that supplied with the kit but a range prepared with recombinant human IFN-α_{2a}. The following seven standard concentrations were included: 0, 3.2, 6.5, 13, 26, 52, and 104 IU/ml.

(ii) Biological method. The biological method described by Lebon et al. (10, 11) measures the IFN-α in a solution by measuring the amount of protection from VSV afforded to a continuous line of bovine renal cells (MDBK cells).

The biological assay was performed in 96-well microtitration plates. The samples were placed in the first row of the plate (the first well being reserved for the standards).

Eight serial twofold dilutions were carried out in basal Eagle's medium supplemented with 10% fetal calf serum. An aliquot of 0.1 ml of a suspension of MDBK cells (150,000 cells per ml) was placed in each well. After incubation for 18 h at 37°C, a confluent cell monolayer was obtained. The culture supernatant was drawn off and replaced by a suspen-

TABLE 1. Results obtained by RIA and the biological assay method

Placenta no.	TR (%)[a]	Fetal outlet sample no.	Titer (IU/ml) by:		Maternal inlet sample no.	Titer (IU/ml) by:		Maternal outlet sample no.	Titer (IU/ml) by:	
			RIA	BM[b]		RIA	BM		RIA	BM
1	0	1	0	0				3[c]		
	0	2	0	0				4[c]		
	23.4	6	0	0				5[c]		
	44.4	7	0	0	7	4,181	8,000	6	775	2,000
	43.3	8	0	0				7	1,131	4,000
	41.6	9	0	0	9	4,203	6,000	8	1,969	4,000
	46.6	10	0	0				9	2,062	4,000
	43.4	11	0	0				10	2,007	4,000
	43.0	12	0	0	12	4,354	4,000	11	1,595	4,000
								15	1,652	3,000
2			0	0	1					
	0	2	0	0	2	0	0	2	0	0
	0	3	0	0		0	0	3	340	180
	0	4	0	0				4	984	1,000
	44.6	5	0	0	5	3,838	4,000	5	1,580	2,000
	43.3	6	0	0	7	2,887	4,000	7	2,024	3,000
	32.7	8	0	0						
	32.6	9	0	0	9	3,144	6,000	9	1,484	4,000
	33.1	11	0	3	11	2,336	6,000	11	1,676	3,000
	37.6	12	10	6	13	3,634	4,000	13	1,015	4,000
	44.2	15	0	0	15	4,176	2,000	15	2,432	4,000
3	0	2	0	0	5	1,160	4,000			
	0	4	0	0	9	4,480	4,000	9	2,840	3,000
	25.0	6	0	0	13	4,720	6,000	13	3,360	3,000
	26.8	8	0	0						
	26.9	10	0	0						
	28.1	12	0	0						
	29.9	14	0	0						
4	38.1	6	0	0	1	0	3			
	37.6	8	0	0	5	3,160	2,000			
	38.9	10	0	0				9	1,920	2,000
	45.3	12	0	0	13	2,520	1,500	13	1,760	2,000
	43.7	14	0	0						

[a] The TR is for tritiated water.
[b] BM, biological assay method.
[c] Results obtained with the second and third assays. For the results of the first assay, see Table 2.

sion of virus in basal Eagle's medium without fetal calf serum; 100 50% tissue culture infective doses ($TCID_{50}$s) of VSV were placed in each well in a volume of 0.1 ml. After an additional 24 h of incubation, the cytopathic effects were estimated. The greatest dilution of sample that still had a protective effect on the cells was evaluated by comparison with the standard dilution range. For each reaction, the following controls were included: two cell controls, two virus controls at dilutions of the viral suspension of 10^{-1}, 10^{-2}, and 10^{-3}, and two controls to check for the absence of any protective effect of Biotrol 00.

RESULTS AND DISCUSSION

The TR of the tritiated water showed the integrity of the placental cotyledon. These TRs were nil before the simultaneous introduction of tritiated water and recombinant human IFN-α_{2a}. Later, tritiated water crossed over the placenta. At steady state, the TR of the tritiated water ranged between 25 and 47%, values which are in the allowed interval (17).

During the experiment, all samples were assayed by the biological method and some were assayed by RIA. The results (Table 1) are for samples assayed by both methods.

The double titration showed that IFN-α did not cross the placenta under the experimental conditions chosen for the present study. The IFN-α concentration was nil in all but 2 of the 31 fetal samples (Table 1). Fetal samples 11 and 12 of placenta 2 gave the only two nonzero readings of a total of about 80 readings for the whole experiment. The titer of fetal sample 11 was 3 IU/ml by the biological assay; this result was corroborated by the RIA (0 ± 3 IU/ml); fetal sample 12 had a titer of 6 IU/ml by the biological assay and a titer of 10 IU/ml by RIA; these concentrations were very much lower than those at the maternal inlets (about 4,000 IU/ml). These transitory results can probably be explained by a minute vascular tearing in the placenta brought about during manipulation, but it was rapidly sealed since the next fetal sample tested (sample 15) no longer contained any IFN-α.

Because the accuracy of the RIA was higher in the middle of the calibration curve than at the ends, a known amount of recombinant human IFN-α_{2a} (50 IU/ml) was added to certain fetal samples. No significant difference was found with respect to the 50 IU that was added, within the background noise limits, confirming the total absence of IFN-α in the fetal samples.

TABLE 2. Results obtained with the first, second, and third RIA and biological assay[a]

Maternal outlet sample no.	Titer (IU/ml) at:					
	First titration		Second titration		Third titration	
	RIA	BM	RIA	BM	RIA	BM
3	30,625	1	0	0	0	0
4	26,666	1	0	0	0	0
5	0	0	0	0	0	0

[a] Results are for placenta 1. BM, biological assay method.

The amounts of IFN-α found at the maternal inlets by RIA were near the expected 4,000 IU/ml; the values obtained by the biological method were almost always slightly higher. Note that perfusion of recombinant human IFN-α_{2a} started at maternal inlet sample 5 (25th min) (Fig. 1) in all the placentas; this accounted for the zero values until that time.

For all assays, the values were about 1,000 IU/ml lower at the maternal outlet than at the inlets; this amount could be retained or degraded during passage through the placenta. As expected, maternal outlet samples 1, 2, 3, and 4 had zero values; from maternal outlet sample 5 (start of recombinant human IFN-α_{2a} perfusion) the values rose, with a few interesting exceptions (Table 2).

For placenta 1, before the start of IFN-α perfusion, very high levels of IFN-α were detected by RIA in maternal outlet samples 3 and 4; the biological method showed only slightly positive results.

These samples were assayed twice more after undergoing two freeze-thaw cycles (freezing to −30°C between each assay); the results showed zero values by both techniques (Tables 1 and 2). It seems, therefore, that an endogenous substance that is similar to IFN-α was detected by the Ab used in the RIA. It gave little protection to the MDBK cells and was inactivated by freezing and thawing. Could it be a placental secretion, as already observed (3), or was it present in the maternal circulation before delivery?

The return of maternal outlet sample 5 to zero values was established before the start of recombinant human IFN-α_{2a} perfusion in the placenta in question (placenta 1).

For placenta 2, IFN-α was found in the maternal outlets before the start of recombinant human IFN-α_{2a} perfusion (Table 1). The correlation between the two assay methods was good, unlike the situation found for placenta 1, and the titers were lower. Freezing of the samples did not significantly alter the titers found in subsequent assays. The absence of IFN-α in maternal outlet sample 2 suggests an endogenous placental origin for the IFN-α that was detected.

Analysis of the correlation of the steady-state results between the two methods and for the four placentas was carried out. The correlation between the two methods was good (correlation coefficient, 0.91). The mean values of the steady-state results for maternal inlets, maternal outlets, and fetal outlets were plotted (Fig. 2). The slope of the plot obtained was slightly greater than 1; this was because the values found at the maternal inlets and maternal outlets were always a little higher by the biological method. This also indicates that the plot does not go through the origin, even though the correlation at the fetal outlets, almost all zero values, was excellent between the two methods.

Sensitivity. The biological method proved to be much more sensitive in detecting low levels of IFN-α. It was able to detect a concentration of 1 IU/ml. The accuracy of the RIA kit, as indicated by the manufacturer, is 10% at 8 IU/ml, and this value increases at lower concentrations. We considered

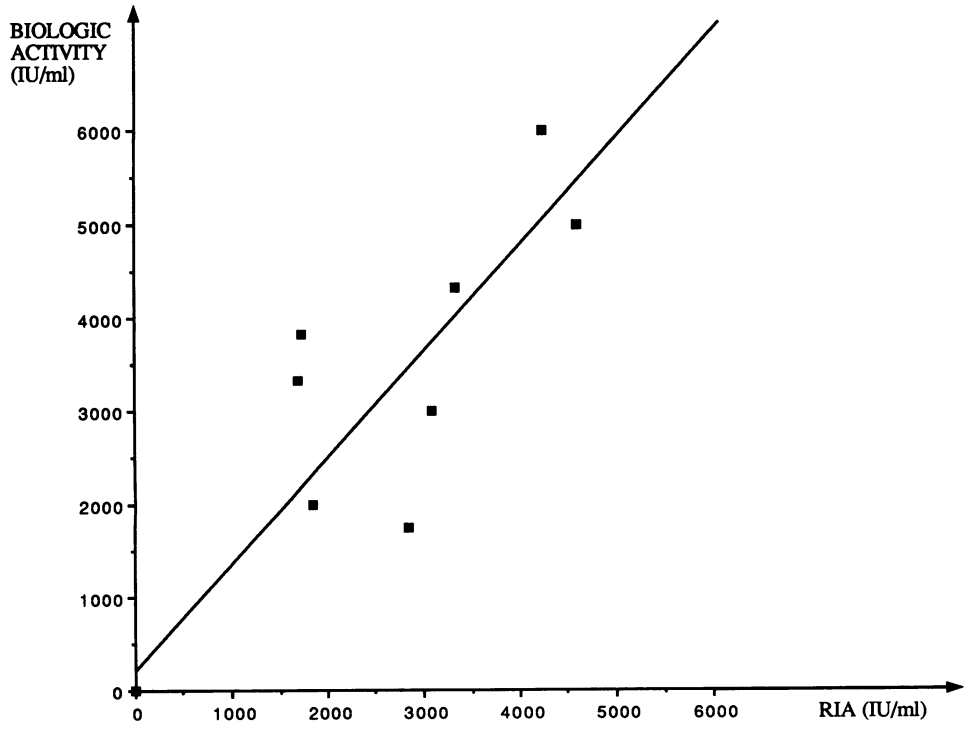

FIG. 2. Correlation plot obtained from the steady-state results for the four placentas ($y = 209.39 + 1.14x$; $r = 0.91$).

TABLE 3. Reproducibility of the RIA studied on the four RIA kits used

Recombinant human IFN-α_{2a} solution titer (IU/ml)	Measured titer (IU/ml)[a]	Coefficient of variation (%)
3.2	3.0 ± 0.5	17
6.5	7.0 ± 0.8	11
13	12.4 ± 1.0	8
26	25.1 ± 2.2	9
104	106 ± 6.0	6

[a] Values are means ± standard deviations.

the RIA to be accurate to within 3 IU/ml at concentrations of less than 8 IU/ml. At levels of about 4,000 IU/ml, which are reached in the maternal circulation, dilutions of 1/40 were made to bring the assay nearer the middle of the RIA calibration curve.

Specificity. For RIA specificity is based on the antigen-Ab reaction, and for the biological method specificity is based on the reaction cascade that leads to the protection of a population of cells. A discrepancy occurred in only one case for maternal inlet sample 1 in placenta 4, in which RIA detected no IFN-α and the biological method found IFN-α at a titer of 3 IU/ml. During IFN-α perfusion, however (at maternal inlet sample 5), much higher and concordant values were found.

For the biological method, the following controls were systematically placed in each plate: cell viability controls and controls of the total destruction by VSV. The batch of Biotrol 00 used during the assay was also tested by RIA and the biological method to ensure the total absence of IFN-α (no protection of MDBK cells from VSV in the presence of Biotrol 00).

Reproducibility. The two IFN-α assay techniques described here were previously established and tested individually. However, during the experiment described here, owing to the frequent repetition of the analysis of identical samples, a study of the reproducibility was possible. Most of the results presented in Tables 1 and 2 were determined twice by both methods. Under the conditions of preserving the samples described above (rapid handling, freezing, and storage at −30°C), we did not observe any consequent drop in the IFN-α titer, and we never observed the presence of IFN-α during a second assay when the first assay gave a zero reading. We used samples with known amounts of recombinant human IFN-α_{2a}. Eight determinations were carried out during all the experiments with the different RIA kits used (Table 3). The reproducibility of the results given by one RIA kit was studied by the repetition of five determinations on three samples (Table 4).

Because we used twofold dilutions for the biological method, a second assay can give a result that was the same as that obtained at an adjacent dilution (causing a twofold variation). This did not occur in most cases, but it did introduce variability that was greater than that which occurred with the RIA.

The only significant variations observed in the present study concerned maternal outlet samples 3 and 4 of placenta 1 (Table 2), but the question arises as to the exact nature of the substance detected.

Practicality. The biological method involves the culturing of cells and could appear to be more demanding than RIA; it is, however, quite straightforward for normally trained technicians. The disadvantages inherent in the RIA are the requirement for radioactive chemicals along with the limited shelf-life of the radioactive element.

Cost. The cost of the biological method is much less than that of the RIA.

The two assay methods described here study the substance whose titer is to be determined from different angles: antigenicity (recognition by the Ab in the RIA) and biological activity (by the protection of a cell population from VSV). Our results showed a good correlation between the two methods. This type of experiment benefits from the combined use of two techniques having different approaches. The agreement observed between the two methods enables us to confirm the absence of transplacental transfer of IFN-α under the experimental conditions chosen.

TABLE 4. Reproducibility of the RIA studied with one RIA kit

Recombinant human IFN-α_{2a} solution titer (IU/ml)	Measured titer (IU/ml)[a]	Coefficient of variation (%)
3.2	3.1 ± 0.3	10
26	25 ± 2	8
104	102 ± 4	4

[a] Values are means ± standard deviations.

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