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Interferon synthesis by human colostral leucocytes

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SUMMARY The antiviral potential of human colostral leucocytes was assessed by their capacity to produce interferon. Leucocytes cultured from colostrum were stimulated by mitogens or Newcastle disease virus (NDV) to produce interferon which, by metabolic and physicochemical criteria, corresponded to normal human leucocyte interferon. Prepartum cells produced higher levels than postpartum cells. Colostral cells were less efficient producers than blood leucocytes.

Human colostrum and breast milk contain inhibitors for a variety of viruses (Sabin and Fieldsteel, 1962; Shortridge *et al.*, 1974; Matthews *et al.*, 1976). The presence of interferon in human colostrum or milk has not been conclusively demonstrated, although colostral leucocytes are known to be able to synthesise interferon-like material in response to stimulation (Emödi and Just, 1974; Lawton and Shortridge, 1977).

In view of the current interest in the function of these cells and their possible contribution to neonatal defences in breast feeding, we have investigated further the capacity of colostral cells to synthesise interferon when stimulated by mitogens and inactivated NDV. We also report the results of our characterisation of this substance.

Materials and methods

Preparation and culture of colostral cells. Colostrum samples were obtained from 26 women, mainly Chinese, by manual expression into sterile plastic universal bottles. 11 samples were prepartum and 15 were 2 to 5 days postpartum. Samples were diluted 1:1 in Hank's balanced salt solution (HBSS), centrifuged at $110 \times g$ for 10 min and the pellet washed twice in HBSS before counting the cells in a haemocytometer. Total cell counts in colostrum ranged from $0{\cdot}6$ to 11×10^9/l (600 to 11 000/mm^3) mean $3{\cdot}37 \times 10^9$/l (3370/mm^3). Differential counts were performed on stained cytocentrifuge preparations; macrophages made up 11–94% (mean 57%), polymorphonuclear leucocytes (PMNs) 2–87% (mean 33%), and lymphocytes 1–39% (mean $9{\cdot}6$%).

After counting, the cells were resuspended at a concentration of 2×10^9 cells/l (2000/mm^3) in leucocyte growth medium (LGM) comprising RPMI-1640 (Gibco) supplemented with 20% heat-inactivated human AB serum, glutamine 2 mmol/l, penicillin 100 U/ml, and streptomycin 100 μg/ml. One ml volume of cell suspension was dispensed into Nunc tissue culture tubes; the total number of cells obtained from the colostrum sample (usually about 15×10^9/l (15 000/mm^3)) limited the number of replicate cultures that could be performed. Mitogens known to stimulate leucocyte interferon synthesis were added to the cultures in predetermined optimal doses; PHA-P (Difco) 5 μl/ml, Conconavolin-A (Sigma) 40 μg/ml. Cells were also preincubated for 30 min with an optimal dilution of a lentogenic (avirulent) strain of NDV (D2/75) and then washed 3 times before resuspending in LGM for culture. Each experiment included an unstimulated control culture. Cultures were incubated at 37°C for 72 hours. The supernatants were harvested and replicate supernatants pooled before being assayed for interferon activity. Supernatants were routinely stored frozen at −20°C, but for periods of less than 24 hours they were held at 4°C.

Interferon assay. The assay was based on that of Havell and Vilček (1972) using human diploid foreskin fibroblasts in Linbro micro T/C plates. Fibroblasts were challenged with vesicular stomatitis virus (VSV) after 24-hour exposure to culture supernatants and the titre of interferon was expressed as the reciprocal of the highest protective dilution. A standard interferon preparation (13 000 NIH units/

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ml) consistently gave a titre of 50 000. Samples were titred at trebling dilutions beginning at 1/5 and assayed in duplicate.

Dialysis of samples before assay did not alter the interferon titre but it did reduce the cytotoxic effect on fibroblasts which was sometimes observed at low dilutions (1/5 or 1/15). Therefore predialysis of samples at pH 2 was adopted as standard procedure (see below, acid dialysis). Samples containing Con-A tended to be more toxic to the fibroblasts at low dilutions and this effect was not removed by dialysis. Consequently Con-A was often omitted and Con-A supernatants were not used in the characterisation studies even though it tended to induce higher interferon levels than PHA or NDV.

Comparison of colostral cells and autologous blood leucocytes. In 7 cases autologous blood leucocytes were cultured in parallel with the colostral cells to compare their capacities to synthesise interferon-like substances *in vitro*.

Blood lymphocytes were separated from heparinised blood over Ficoll/Hypaque, washed 3 times in BSS, counted and resuspended in LGM at 2×10^9 cells/l ($2000/mm^3$). The suspensions contained 5–15% monocytes. The blood mononuclears were stimulated with PHA and NDV as described for colostral cells. After 72 hours' incubation the supernatant was harvested and assayed for interferon activity.

Characterisation studies. High titre supernatants of PHA- or NDV-stimulated cultures were pooled and studied as follows:

Metabolic characterisation

This was based on the fact that the protective effect of interferon requires intact mechanisms of RNA and protein synthesis in the cells on which protection is to be conferred (Ng and Vilček, 1972).

To inhibit RNA synthesis fibroblasts were pretreated with actinomycin D (0·5 and 1·0 μg/ml in culture medium) for 30 min at 37°C then washed 3 times in HBSS before adding the pooled supernatant. To inhibit protein synthesis cycloheximide was diluted in culture medium to 100 μg/ml and this medium was used as diluent for the pooled supernatant. Cells exposed to cycloheximide were washed 3 times in HBSS before virus challenge. Supernatant was also assayed on control cells not exposed to metabolic inhibitor. Each group included appropriate positive and negative controls for virus cytopathic effect.

Physiocochemical characterisation

Supernatant aliquots were treated as described below before assay.

Acid dialysis. Supernatants were dialysed against 0·1 mol/l KCl, pH 2, at 4°C for 18 hours, then against phosphate buffered saline (PBS), pH 7·4, for 4 hours. Control supernatants were dialysed for 22 hours against PBS only.

Freeze-thaw, heat, and trypsinisation. Respective aliquots were frozen to −70°C and thawed 5 times, heated to 56°C for 30 min, or incubated in the presence of trypsin (1·25 mg/ml) at 37°C for 5 hours. A control aliquot was kept at 4°C.

Detection of sialic acid residues. Pooled supernatant of known titre was applied to a column of immobilised neuraminidase at pH 4·5. The column was developed by elution with 0·1 mol/l/acetate buffer in 0·5 mol/l NaCl, pH 4·5, followed by 0·1 mol/l bicarbonate in 0·5 mol/l NaCl, pH 9·0. The eluted protein peaks (0D 280 nm) were collected and tested for interferon activity (Fung and Ng, 1978).

In the interferon characterisation experiments supernatants were titred at trebling dilutions.

Results

Table 1 shows the geometric mean titres of interferon in the colostral cell cultures. Two PHA supernatants (>135) were not titred to the endpoint; these were taken as 135 and all negative samples (<5) were taken as 1 for purposes of calculation. The highest interferon titre obtained was 640 in a Con-A stimulated culture from a prepartum sample. The combined results (pre- and postpartum) show that Con-A gave the highest mean interferon activity, while NDV, although less efficient than Con-A, was a more efficient inducer than PHA. All unstimulated control cultures were negative for interferon activity (titre <5).

Prepartum colostral cells produced higher interferon levels than postpartum cells; this was so for

Table 1 *Interferon activity in colostral cell culture supernatants*

	PHA stimulated	*Con-A stimulated*	*NDV stimulated*	**All inducers*
Prepartum	11 (1–320)	59 (1–640)	38 (15–135)	21 (1–640)
	n = 11	n = 3	n = 7	n = 21
Postpartum	7·6 (1–135)	24 (1–135)	12 (1–45)	11 (1–135)
	n = 15	n = 6	n = 8	n = 29
All cultures (pre- and post-partum)	9·7 (1–320)	32 (1–640)	19 (1–135)	
	n = 26	n = 9	n = 15	

The results are expressed as geometric mean titres of 72-hour culture supernatants with titre ranges in parentheses. Differences between mean titres of pre- and postpartum cultures did not reach significance at the 5% level (2-tailed Student's *t* test). Unstimulated control cultures were negative (titre <5) in all experiments.
*Pooled data for the 3 inducers.

each inducer but even when data were pooled (Table 1) the difference was not significant at the 5% level ($0{\cdot}15>P>0{\cdot}1$). This difference could not be explained by the relative proportions of cell types in the cultures; the mean percentages of macrophages, lymphocytes, and PMNs in prepartum and postpartum colostrum were very similar and the overall data showed no clear correlation between interferon titres and the proportions of cell types in the original culture inocula.

Colostral cells and autologous blood leucocytes. The geometric mean titres of interferon from these paired cultures are set out in Table 2. The capacity of blood mononuclears to synthesise interferon in response to NDV stimulation was significantly higher than that of colostral cells ($P<0{\cdot}025$), whereas the difference in response to PHA did not reach a level of significance ($P>0{\cdot}15$).

Table 2 also shows that NDV was clearly a more efficient inducer of interferon in blood leucocytes than was PHA (ratio, 8·5:1), whereas colostral cells demonstrated little difference in response to these two inducers.

Interferon characterisation. The results of the characterisation studies are summarised in Table 3. Inhibitors of RNA and protein synthesis both abrogated the protection of fibroblasts by the culture supernatants, consistent with the protective effect being due to interferon.

Activity was not dialysable; it was stable at pH 2 and destroyed by trypsin. PHA-induced activity was stable to freeze–thaw and relatively heat stable. On the other hand, NDV-induced activity was relatively freeze–thaw stable and heat labile.

Interferon activity induced by NDV bound to a column of immobilised neuraminidase at acid pH via its N-acetyl-neuraminic acid residues and could be recovered essentially without loss of activity by elution at pH 9·0.

Table 2 *Interferon activity in colostral cell and autologous blood leucocyte cultures*

	PHA-stimulated		*NDV-stimulated*	
Colostral cells	16 (1–135)	$P>0{\cdot}15$ ($n=7$)	18 (5–45)	$P<0{\cdot}025$ ($n=6$)
Autologous blood leucocytes	10 (1–45)		85 (15–415)	

The results are expressed as geometric mean titres of 72-hour culture supernatants with titre ranges in parentheses. P values were computed by the Student's *t* test for correlated data. Unstimulated control cultures were negative (titre <5) in all experiments.

Discussion

Recent studies have shown that leucocytes in colostrum may play an important role in transferring specific and nonspecific host resistance factors to the neonate (Murillo and Goldman, 1970; Ahlstedt *et al.*, 1975; Parmely *et al.*, 1976; Pitt *et al.*, 1977; Schlesinger and Covelli, 1977). Our results confirm that colostral leucocytes can synthesise interferon under appropriate conditions and that their capacity to do so is comparable with that of blood leucocytes (Emödi and Just, 1974).

Interferon protects a cell by first inducing the synthesis of an antiviral protein (Joklik, 1977) and this process is dependent on intact mechanisms of RNA and protein synthesis (Ng and Vilček, 1972). It was found that the antiviral effect observed in our experiments was dependent on (1) pretreatment of cultures with supernatant, and (2) intact macromolecular synthesis of the fibroblasts. It was further shown that the antiviral substance was nondialysable, resistant to treatment with acid, stable to freeze–thaw, destroyed by trypsin, and it appeared to have sialic acid residues which bound it to immobilised neuraminidase. These are all known properties of interferon.

Surprisingly, this interferon was unlike classical human leucocyte interferon (type I) in being relatively heat labile. Haahr *et al.* (1976) showed that the presence of fully differentiated macrophages resulted in the production of heat-labile (type II) interferon by human lymphocytes. Therefore the high proportion of mature macrophages in colostral leucocyte cultures may account for heat-labile interferon synthesis. Because our colostral cells contained a mixture of lymphocytes (usually <10%) and macrophages, it is difficult to draw any conclusions about

Table 3 *Metabolic and physicochemical characterisation of interferon activity*

	Actinomycin-D 0·5 µg/ml	*Cycloheximide 100 mg/ml*	*Dialysis pH 2*	*Freeze-thaw*	*Heat*	*Trypsin*	*Sialic acid residues*
PHA-induced	Not done	+	−	−	±	+	Not done
NDV-induced	+	+	−	±	+	+	Present

Supernatants titred at trebling dilutions.
− Denotes stability; ± denotes reduction in activity of one dilution; + denotes reduction in activity of more than one dilution (or abrogation of interferon effect by metabolic inhibitors).

the principal cell of origin of the interferon activity. Human interferon is known to be heterogeneous with respect to the cell of origin and to the type of inducer (Vilček *et al.*, 1977); it is likely, therefore, that the active material in our cultures represented a mixture of 'interferons'.

It is of interest that prepartum colostrum leucocytes showed a greater capacity to produce interferon than did postpartum cells. This difference could not be related to the very small differences in proportions of cell types in the cultures; the reason for it remains unknown.

Emödi and Just (1974) and Matthews *et al.* (1976) were unable to detect free interferon-like activity in human colostrum or breast milk. We have been able to detect interferon-like activity in a small proportion of the samples of colostrum and breast milk which we have tested (unpublished observations) and work is in progress to characterise it. It is not yet clear whether this activity is related to infection in the mother.

The important implication of our findings is that since colostral leucocytes under appropriate conditions can produce interferon, they may thereby confer on the suckling infant protection against viral infection.

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