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Interferon production and lymphocyte stimulation in human leucocyte cultures stimulated by *Corynebacterium parvum*

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SUMMARY

Killed *C. parvum* organisms stimulated a lymphoproliferative response in human peripheral leucocyte cultures of both adult and cord blood origin. They also induced high titres of interferon in cultures of adult leucocytes, but there was no correlation between the degree of lymphocyte stimulation and of interferon production. A considerable variability between donors was seen in both assays. The amount of interferon produced in *C. parvum*-stimulated cultures was considerably higher than that stimulated by the T-cell mitogens PHA and Con A and that induced by LPS. The anti-viral protein induced by *C. parvum* fulfilled the criteria of interferon and appeared to represent type II interferon.

INTRODUCTION

C. parvum represents a strong stimulant of the lymphoreticular system (Halpern *et al.*, 1964) and has been successfully used for the immunotherapy of experimental tumours in animals (for review see Scott, 1974). Following the first report by Israel & Halpern (1972), a large number of therapeutic trials have been performed using *C. parvum* in cancer patients (Halpern, 1975). We have recently shown that *C. parvum* also protects mice against viral-induced encephalitis (Kirchner, Hirt & Munk, 1977c) and have subsequently demonstrated that it induces the *in vitro* production of interferon in mouse spleen cells (Kirchner *et al.*, 1977a,b). We report here that *C. parvum* represents an interferon inducer in cultures of human peripheral leucocytes. During the progress of this work similar data have been described in an abstract by Epstein & Sugiyama (1977).

MATERIALS AND METHODS

Leucocyte cultures. Heparinized venous blood from unselected healthy donors, or heparinized cord blood samples, were mixed with equal amounts of 0·9% saline and the mixture was carefully layered over a pre-warmed Ficoll–Hypaque preparation (Lymphoprep®, obtained from Molters, Neckargemünd, F.R.G.) in 50 ml tissue culture tubes (No. 2070, Falcon Plastics, Oxnard, California). These were centrifuged at 400 *g* at room temperature for 30 min and the cells from the interphase of the gradient were collected and washed three times in saline. Cultures of this population which contained 10–20% monocytes and about 1% granulocytes will subsequently be referred to as leucocyte cultures. The cells were cultured in medium RPMI 1640 supplemented with 2 mM glutamine, 100 iu/ml penicillin and 100 μg/ml streptomycin (all reagents from Grand Island Biological Co., Grand Island, New York). As a serum supplement, 10% of one batch of AB serum pooled from five donors, which was stored frozen, was used in all experiments.

C. parvum or the mitogens were added at the beginning of the cultures. The source of the mitogens phytohaemagglutinin (PHA), concanavalin A (Con A) and *E. coli* endotoxin (LPS) have been previously detailed (Kirchner *et al.*, 1976). Formalin-killed *C. parvum* (strain designation CN 6134) were kindly provided by Dr M. T. Scott (Department of Experimental Immunobiology, The Wellcome Research Laboratories, Beckenham, Kent, UK). These bacterial suspensions contained 0·01% thiomersal as a preservative. Therefore, before use, 50 ml of bacteria were taken up in 50 ml of saline in 50 ml tubes (Falcon) and centrifuged for 15 min at 1000 *g*. This centrifugation was repeated three times, and after the third centrifugation the bacteria were taken up in medium RPMI 1640.

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For induction of interferon-containing supernatants, the leucocytes were cultured for 1 day at 37°C in 3·0 ml quantities in 16 × 125 mm tissue culture tubes (Falcon) at a density of 10^6 cells per ml. Extensive preliminary testing had revealed that optimal levels of interferon were already found after 24 hr and that there was little or no increase during the next few days of culture. The tubes were centrifuged and the cell-free supernatants were frozen at −70°C until testing.

Parallel cultures were set up in microtitre plates to assess the lymphoproliferative response by the incorporation of tritiated thymidine ([^{3}H]TdR) exactly as described (Kirchner *et al.*, 1976, 1977a). In this assay, cultures were set up in quadruplicates and the mean ct/min were calculated. Since the standard errors of the mean were always below 5%, they were not usually given. In some experiments lymphocyte stimulation was expressed by the stimulation index (SI):

$$SI = \frac{\text{mean ct/min of stimulated cultures}}{\text{mean ct/min of unstimulated control cultures}}.$$

Interferon assay. Leucocyte culture supernatants were tested for their ability to interfere with the replication of vesicular stomatitis virus (VSV) in human foreskin fibroblasts (HFF). Confluent monolayers of HFF in Leighton tubes (1906-16125, Bellco, Vineland, New Jersey) were pre-incubated for 18 hr with the supernatants, diluted supernatants or with interferon standards, thoroughly washed and infected with VSV at a multiplicity of infection of 1·0. After infection, the cells were cultured for 24 hr, frozen and thawed and the cellular material was removed by centrifugation (700 *g*, 10 min). Viral titres of the supernatants were tested in a viral plaque assay. This assay was performed in ninety-six well microtitre plates with flat-bottomed wells (Microtest II, No. 3040, Falcon). Confluent monolayers of a permanent line of monkey kidney cells, RITA, were infected with ten-fold serial dilutions of the test material (every dilution was tested in replicate wells) and after 45 min, 0·2 ml of medium containing 1% carboxymethylcellulose (Serva, Heidelberg, F.R.G.) were added. The plates were incubated for 48 hr, and after decanting of the medium were fixed and stained. Virus titres were determined by counting the plaques and were given as plaque-forming units (PFU) per ml. This type of two-step interferon test gave highly reproducible results when multifold samples of one supernatant were compared.

We used two types of interferon standards. One was induced in human leucocytes by Sendai virus (reference number G-023-901-527, NIAID, National Institutes of Health, Bethesda, Maryland). Another standard was obtained from the Behringwerke (Marburg, Lahn, F.R.G.), derived from human leucocyte cultures treated by Newcastle disease virus. When the two standards were compared in our test system, the results compared well. About 0·5 iu of each standard were required to inhibit the yield of VSV in HFF by 50%. Standard curves were established from which the interferon content of each test sample could be determined.

RESULTS

Stimulation of lymphoproliferation and of interferon production by C. parvum

Cultures of peripheral leucocytes were established from sixteen unselected healthy adult individuals. As can be seen in Table 1, all of these showed a positive lymphoproliferative response to *C. parvum*. However, the degree of stimulation was quite variable, with ratios of stimulation between 4 and 235. Leucocytes from five different cord bloods also responded to *C. parvum* with a marked lymphoproliferative response.

High titres of interferon could be detected in the supernatants of adult leucocyte cultures after treatment with *C. parvum* (Table 1). Again, a considerable degree of variation was observed when leucocyte cultures from different individuals were compared. The amount of interferon in supernatants of *C. parvum*-stimulated cord blood cultures was comparably small.

When the degree of lymphocyte stimulation and of interferon production was compared in parallel cultures of the same individual, as in the experiment shown in Table 1, no correlation could be detected. In this regard, it is also noteworthy that the optimal dose for the induction of interferon was consistently ten times higher than the dose inducing optimal lymphoproliferation (complete dose–response curves not shown).

Comparison between C. parvum, PHA and Con A

As others have shown (Wheelock, 1965; Falcoff, 1972), the T-cell mitogens PHA and Con A also induced the production of interferon in human leucocyte cultures (Table 2). It has been a consistent finding in our laboratory that in all instances where the dose-responses and kinetics were compared, the amount of interferon in cultures stimulated by the T-cell mitogens was considerably lower than that found in *C. parvum*-stimulated cultures. LPS hardly stimulated human peripheral leucocyte cultures and produced only moderate amounts of interferon (Table 2).

TABLE 1. Interferon production and lymphocyte stimulation in cultures of human peripheral leucocytes of adult and cord blood origin

Adult Donor	Amount of interferon*	Lymphocyte stimulation†
(1)	300	4
(2)	360	9
(3)	3500	20
(4)	1010	146
(5)	3300	66
(6)	3300	9
(7)	3900	235
(8)	170	12
(9)	420	77
(10)	120	33
(11)	1100	130
(12)	220	131
(13)	160	69
(14)	2000	9
(15)	>10000	8
(16)	420	66
Cord blood		
(1)	150	13
(2)	90	27
(3)	80	7
(4)	70	15
(5)	230	75

* Expressed as interferon units/ml. Standard curves of reference interferon were used for calculation as described in the Materials and Methods section. Control cultures without added *C. parvum* always produced less than 10 units/ml.

† Stimulation ratio. Note that the optimal dose of *C. parvum* for interferon induction was 140 μg/ml, while the dose for optimal lymphocyte stimulation was 14 μg/ml.

TABLE 2. Lymphocyte stimulation and production of interferon in cultures of human peripheral leucocytes

Stimulant	DNA synthesis*	Titre of interferon (iu/ml)†
None added	321	10
PHA (6·2 μg/ml)	125699	90
Con A (3·1 μg/ml)	106065	105
LPS (10 μg/ml)	650	35
C. parvum (1·4 μg/ml)	41532	(140 μg/ml) 6530

* Mean ct/min of [^{3}H]TdR incorporated on day 5 of culture during the final 4 hr of incubation. Results are means of quadruplicate cultures, standard errors of the mean were all below 5% and are not given.

† The cell-free supernatants of the leucocyte cultures were recovered after 1 day and tested for their capacity to inhibit the replication of VSV in HFF.

TABLE 3. Properties of interferon by *C. parvum* in human leucocyte cultures in comparison with interferon induced by Newcastle disease virus

Interferon property	Inducing Agent *C. parvum*	NDV*
Sensitivity to:		
Trypsin†	+	+
56°C (1 hr)	+	−
pH 2·0 (24 hr)	+	−
Dialysis‡	−	−
Centrifugation (100,000 *g*, 1 hr)	−	−
Species specificity	+	+
Virus specificity	−	−
Direct neutralizing effect on virus§	−	−

* Interferon standard derived from human leucocytes treated by NDV (Behringwerke, F.R.G.).

† The two interferon preparations were incubated for 1 hr at 37°C in the presence of 0·2 mg/ml of trypsin. Subsequently foetal bovine serum was added to stop the reaction.

‡ Interferon preparations were dialysed against Hanks's balanced salt solution at 4°C for 48 hr with two changes of the salt solution.

§ In order to demonstrate an anti-viral effect, the cells had to be pre-treated with interferon for several hours. No effect was seen when interferon was added to the monolayers of HFF cells at the time of infection with VSV.

Properties of interferon induced by C. parvum

Some of the properties of the anti-viral activity found in the supernatants of *C. parvum*-treated human leucocytes are listed in Table 3. These criteria justify the conclusion that this activity is indeed interferon. The properties are similar to those recently attributed to type II interferon. *C. parvum*-induced interferon was sensitive to trypsin treatment, to heating at 56°C and treatment at pH 2·0. It was not lost after dialysis or centrifugation. Species specificity between human and mouse cells was observed since the replication of VSV in mouse L cells could not be inhibited. However, replication of HSV in human cells could be equally inhibited as the replication of VSV. There was no direct neutralizing effect of the *C. parvum*-induced supernatant on these viruses.

For comparison we also studied an NDV-induced human (type I) interferon, the properties of which are also listed in Table 3.

DISCUSSION

The list of the heterogeneous substances that induce interferon is fairly extensive and steadily increasing. A tentative classification of interferon inducers has recently been proposed (Ho & Armstrong, 1975). As type II production, a type of interferon production has been classified, resulting from specific and non-specific activation of lymphocytes and macrophages. The interferon, which is found in human peripheral leucocyte cultures stimulated by *C. parvum,* by definition represents type II interferon and the physicochemical properties listed in Table 3 are similar to those usually ascribed to type II interferon (Ho & Armstrong, 1975). However, characterization of proteins by heat and pH sensitivity is probably not very meaningful.

From our data it appears that *C. parvum* is considerably more potent in inducing interferon production than the T-cell mitogens PHA and Con A. This was consistently found in many experiments where the conditions of induction by PHA and Con A were varied (unpublished results). Recently, we have obtained similar data comparing *C. parvum* and various mitogens for their ability to induce interferon in mouse spleen cell cultures (Kirchner *et al.*, 1977b).

LPS which is a B-cell mitogen for mouse spleen cells is not mitogenic for human leucocytes (Peavy, Adler & Smith, 1970). In our experiments, it was a poor inducer of interferon production in human leucocyte cultures. This strongly argues against the possibility that LPS contamination accounted for the interferon-inducing capacity of *C. parvum*. Furthermore, the batch of *C. parvum* which we used has been found to be free of LPS by the limulus assay (M. T. Scott, personal communication).

From our knowledge of the literature, it appears that *C. parvum* induced relatively high amounts of interferon when compared with known inducers of type II interferon. Type II interferon is just one example of the numerous lymphokines and monokines (David, 1973). These have been classified by effects that can be measured in a variety of biological assays. It remains to be determined, however, whether these different activities may only be due to a small number of molecules with a large spectrum of activities. Some lymphokines, such as migration inhibitory factor, appear to be inseparable from type II interferon (Younger & Salvin, 1973). Thus type II interferon may have important implications as a lymphocyte- or macrophage-derived regulator molecule which affects both cellular functions and the replication of viruses in these cells. To further establish this hypothesis, purification and characterization of this type of interferon will be required. *C. parvum* may represent a practical and useful inducer to stimulate large enough quantities of interferon for this purpose.

The main interest in *C. parvum* stems from its effect as an immunostimulant in experimental tumour systems (Scott, 1974). In view of the large number of clinical trials with *C. parvum* which have been recently initiated, it is important to collect further data on the effects of *C. parvum* on immunocompetent cells. In mice, *C. parvum* represents a weak B-cell mitogen (Zola, 1975). We have found that *C. parvum* represented a lymphocyte stimulant in cultures of human peripheral leucocytes, and in unpublished studies (in collaboration with H. Rühl), we have shown that it was a T-cell stimulant in this system. This is in agreement with the data presented by Epstein & Sugiyama (1977). These authors have also found that the cell producing interferon in response to *C. parvum* was a T cell, while from our own unpublished data it appeared that both purified T cells and purified B cells were able to respond to *C. parvum* with production of interferon.

At present, it is not completely clear whether *C. parvum* acted as a mitogen or an antigen in the human leucocyte cultures. All donors tested thus far responded to *C. parvum* with a lymphoproliferative response, as did cultures of cord blood lymphocytes, suggesting that *C. parvum* acted as a mitogen. However, since there is a widespread sensitization of normal individuals to *C. parvum* or cross-reacting bacteria (Wolberg *et al.*, 1977), it cannot be excluded that the observed stimulation represented stimulation by specific antigen.

Interferon has recently been shown to have antiproliferative effects on tumour cells *in vitro* (Gresser *et al.*, 1970) and also to inhibit certain *in vitro* lymphocyte functions, such as stimulation by mitogens (Lindahl-Magnusson, Leary & Gresser, 1972). It has been found that macrophages in spleens of *C. parvum*-injected mice inhibited both the proliferation of tumour cells and mitogen-induced lymphoproliferation (Scott, 1972; Olivotto & Bomford, 1974; Kirchner, Holden & Herberman, 1975). From these analogies it is tempting to speculate that interferon may act as a mediator molecule of such macrophage functions. To further establish this hypothesis, we are presently performing experiments to define the nature of the interferon-producing cell, in both mouse spleen cell cultures and in human peripheral blood cell cultures.

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