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Cytokine release from human peripheral blood leucocytes incubated with endotoxin with and without prior infection with influenza virus: relevance to the Sudden Infant Death Syndrome

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Summary. Previous work with a neonatal ferret model for human SIDS had indicated that inflammation caused by a combination of influenza virus and bacterial endotoxin may be a cause of human SIDS. To determine whether cytokines may be involved in this inflammatory response, levels of interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α were examined, using ELISA assays, in culture supernatants of human peripheral blood leucocytes infected with influenza virus and subsequently incubated with endotoxin. Levels of TNF-α were increased compared to cells incubated with virus or endotoxin alone. Levels of IL-1β were also increased whereas levels of IL-6 were generally not enhanced. Cytokines appeared within 1–2 h of stimulation with virus or endotoxin and increased subsequently to reach maximum titres between 16 and 20 h post treatment. While levels of cytokine were much lower when determined using bioassays rather than ELISA assays, the pattern of increased yields from cells incubated with virus and endotoxin compared with either alone was still evident. The possible importance of these observations for SIDS victims is discussed.

Keywords: influenza virus, endotoxin, LPS, cytokines, interleukin-1, interleukin-6, tumour necrosis factor

The Sudden Infant Death Syndrome (SIDS) or cot death is the most common cause of perinatal mortality in the developed world (Guntheroth 1989). The syndrome is most common in winter (Carpenter & Gardener 1967), there is often a history of upper respiratory tract infection (Valdes-Dapena 1967) and occasionally inflammatory changes are visible at post-mortem (Valdes-Dapena 1967). Infection may thus play a role in some cot deaths and respiratory syncytial virus, parainfluenza virus, rhinovirus, adenovirus and influenza A virus have been associated with this syndrome (Scott *et al.* 1978; Zink *et al.* 1987). Infections with these viruses commonly increase the susceptibility of the respiratory tract to infection with bacteria which have been suggested as a cause of some cot deaths (Sonnabend *et al.*, 1985; Lee *et al.*, 1987) either by the induction of inflammation or by the production of toxins (Morris *et al.*, 1987).

Infection of 1-day-old ferrets with a virulent strain of influenza virus (clone 7a [H3N2] of the reassortant virus A/Puerto Rico/8/34 [PR8]-A/England/939/69) has been used as a model for human infant infection (Collie *et al.* 1980; Husseini *et al.* 1983). Most of the kits died with

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uncomplicated pneumonia but some had an airway obstruction and lack of lung pathology reminiscent of SIDS (Collie *et al.* 1980; Tapp *et al.* 1975). Infection of newborn ferrets with the attenuated PR8 strain also killed some kits (30%) but this was due to secondary bacterial infection (Husseini *et al.* 1983) and, again, airway obstruction and lack of lung pathology was seen in some. No deaths occurred in neonates infected with PR8 and treated with antibiotics to prevent bacterial infection (Husseini *et al.* 1983). Infection of neonatal ferrets with PR8 significantly enhanced their susceptibility to the lethal effects of various toxins including endotoxin (Jakeman *et al.* 1991). Neonates died suddenly without clinical symptoms as in human babies dying of SIDS but there was inflammation in the upper respiratory tract, lung oedema and collapse, and early bronchopneumonia in the toxin and virus-treated animals but not in those treated with toxin or virus alone.

Viruses and toxins alone or in combination have been shown to elicit various inflammatory mediators from human cells including interleukin-1 (IL-1), tumour necrosis factor (TNF) and histamine (Clementsen *et al.* 1988; Lonnemann *et al.* 1989; Nain *et al.* 1990; Gong *et al.* 1991). To determine whether such mediators are involved in the influenza virus and/or toxin induced inflammation, the first step was to see if IL-1β, IL-6 and/or TNF-α were released from human peripheral blood leucocytes following incubation *in vitro* with influenza virus and endotoxin alone or in combination and this is the subject of the present communication.

Materials and methods

Viruses and their assay

PR8 was prepared as described previously (Matsuyama *et al.* 1980) and assayed by the egg technique (Matsuyama *et al.* 1980); titres are expressed as 50% egg infectious doses (EID_{50}).

Bacterial endotoxin

Escherichia coli endotoxin (Sigma; product number L2637) was prepared from strain 055.B5 and purified by phenol extraction and chromatography; it contained <1% protein and <1% RNA.

Treatment of human leucocytes with virus and endotoxin

Human leucocytes were prepared from 'buffy coat' preparations, obtained from the West Midlands Regional Blood Transfusion Service, following 2–3-fold dilution with RPMI-1640 medium. Ten-ml amounts of diluted buffy coat were layered onto 10-ml cushions of Histopaque-1119 (6.0% (w/v) ficoll type 400, 16.7% (w/v) sodium diatrizoate, density 1.119 at 25°C; Sigma) and centrifuged (700 ***g***, 30 min, 22°C) to sediment erythrocytes. Leucocytes were harvested from the supernatant, diluted two-fold with RPMI and sedimented by centrifugation (1000 ***g***, 15 min, 22°C). The cells were resuspended in RPMI-1640, pooled and washed by two further centrifugations (1000 ***g***, 15 min, 22°C) before being resuspended in 10 ml RPMI-1640 and counted. Viability (assessed by trypan blue dye-exclusion) was always >90% and the cell population (differentiated by staining with Giemsa) typically consisted of 68% lymphocytes, 20% neutrophils, 7% monocytes, 1% eosinophils, 1% basophils and 3% other and lysed cells. Cells were then sedimented by centrifugation (1000 ***g***, 15 min, 22°C) and resuspended in RMPI-1640 medium to give 2×10^7 viable cells/ml (Tinsley *et al.* 1987). Infectious virus was added to duplicate 1-ml aliquots of cells to give 100 EID_{50}/ml. After incubation for 1 h in a water bath at 37°C, the cells were sedimented (1000 ***g***, 15 min, 22°C) and the inoculum was removed. The cells were resuspended in RPMI 1640 at 37°C and washed twice (10 ml RPMI-1640, 37°C) before resuspension in 10 ml medium to give 2×10^6 cells/ml. After further incubation at 37°C for 15 h (16 h total incubation), the cells were sedimented (1000 ***g***, 15 min, 22°C) and the supernatant was removed and stored at -70°C until examined for cytokine content.

For samples to be incubated with endotoxin, 10 ng/ml was added either at the time of removal of the viral inoculum (1 h p.i. sample), 1 h after removal of the viral inoculum (2 h p.i. sample) or 3 h after removal of inoculum (4 h p.i. sample). Endotoxin remained in the culture medium throughout the subsequent incubation period. An uninoculated aliquot of cells was included as a control.

TNF-α, IL-1β and IL-6 cytokine ELISA assays

These commercial Quantikine kits were obtained from British Bio-technology (Abingdon, Oxon) and the assay procedures described by the manufacturer were followed.

TNF-α and IL-6 cytokine bioassay

Cytotoxicity for the murine fibroblast L929 line was used to assay TNF (Spofford *et al.* 1974). IL-6 was measured using the murine hybridoma cell line B9, which is unresponsive to IL-1 (Helle *et al.* 1988). An internal standard was included in each assay and results are quoted in pg/ml (TNF) or units/ml (IL-6).

Statistical analysis

The significance of the differences of the results obtained between different treatments was tested using Student's *t*-test for independent samples.

Results

Cytokine stimulation by virus and endotoxin

Human peripheral blood leucocytes generally produced little or no TNF-α or IL-1β spontaneously when incubated *in vitro* for 16 h at 37°C (Table 1). Influenza virus PR8 (100 EID_{50}) induced 2.7 ng/ml of TNF-α whereas those induced by 10 ng/ml endotoxin were lower (1.6 ng/ml). This difference was not significant and in other experiments endotoxin induced more TNF than did virus (for example see Table 4). Incubation of cells with virus and endotoxin enhanced the amounts of TNF-α released, especially when endotoxin was added 1–2 h post viral infection; this enhanced release became significant ($P < 0.05$) although the time at which this occurred varied between experiments. Similar results were obtained with IL-1β where levels again increased but significantly ($P < 0.05$) only when endotoxin was given 1 h after the viral inoculum (Table 1).

On a weight basis levels of IL-6 were about tenfold higher than the corresponding TNF-α and IL-1β levels (Table 1) but any enhancement by endotoxin and virus in combination was not significant ($P > 0.05$).

Table 1. Levels of TNF, IL-1 and IL-6 as determined by ELISA assay in supernatants harvested from human peripheral blood leucocytes after 16 h total incubation following treatment with influenza virus and endotoxin (LPS) for various periods

Treatment	TNF (ng/ml)	IL-1 (ng/ml)	IL-6 (ng/ml)
Cells alone*	0 (0)¶	0.01 (0.01)	0.85 (0.85)
Cells + LPS†	1.59 (0.55)‖	1.31 (0.44)	30.88 (9.51)
Cells + virus‡	2.66 (0.41)	1.32 (0.16)‖	26.79 (6.35)
Cells + virus + LPS (1 h)§	2.99 (0.31)	2.61 (0.22)‖	34.15 (1.50)
Cells + virus + LPS (2 h)§	3.91 (0.19)‖	4.42 (0.87)	35.45 (4.89)
Cells + virus + LPS (4 h)§	2.87 (0.08)	1.76 (1.26)	26.39 (2.41)

* No virus or LPS added.

† LPS (10 ng/ml) was added 1 h after initial cultivation of cells and the cells further incubated for 15 h.

‡ Virus (100 EID_{50}) was incubated with cells for 1 h prior to removal and the cells further incubated for 15 h.

§ Virus (100 EID_{50}) was incubated with cells for 1 h at which time LPS (10 ng/ml) was added (1 h), or LPS was added 1 h after removal of virus (2 h) or 3 h after removal of virus (4 h). Cells were incubated for a total of 16 h.

¶ Standard error of the mean in parentheses.

‖ Significantly different at $P < 0.05$.

Similar results were obtained in two further experiments (data not shown).

Optimum timing of the enhancement

The above experiments were repeated but with samples taken 2, 4, 12, 16, 20 and 28 h after addition of virus. The results for TNF-α and IL-6 are shown in Tables 2 and 3 respectively. There was no measurable spontaneous release of TNF-α from human peripheral blood leucocytes at any time point in this experiment. No TNF-α was found in culture supernatants of cells 2 h after addition of virus or of cells infected with virus for 1 h followed by 1 h incubation with endotoxin (Table 2; 2 h time point). However, low levels of TNF-α (820 pg/ml) were observed when cells were incubated with endotoxin alone for 2 h suggesting that production and release of TNF-α occur between 1 and 2 h post stimulation with endotoxin. At 4 h p.i., both virus and endotoxin alone stimulated TNF-α release but there was no enhancement when given together. From 12–20 h p.i., levels of TNF-α were high (2–6 ng/ml) with yields being highest when 10 ng/ml of endotoxin was added to cells 2 h after viral infection (Table 2). The combination of virus and endotoxin enhanced yields significantly ($P < 0.05$) at all times except 28 h, with the maximum enhancement occurring 20 h post infection.

Again, on a weight basis, levels of IL-6 were considerably higher (up to 50-fold) than the corresponding levels of TNF-α (Table 3). Here, all levels increased from 4 to 20 h post infection after which they levelled out. At all time points, any enhancement by the combination of virus and endotoxin was small and significant ($P < 0.05$) only at 16 h post infection where levels of IL-6 stimulated by LPS alone were anomalous.

Virus and toxin doses

Preliminary experiments (data not shown) indicated that 10 ng/ml endotoxin stimulated maximal yields of TNF-α from human peripheral blood leucocytes. Increasing concentrations up to 200 μg/ml did not induce significantly more TNF-α whereas lower doses such as 250 pg/ml induced 2–3-fold less TNF-α. To examine the possibility that enhancement by virus may be greater when toxin doses were suboptimal, experiments were conducted with an endotoxin dose of 250 pg/ml and a virus dose of 10 EID_{50}. Low amounts of TNF-α were released spontaneously in this experiment but were not increased by incubation with 10 EID_{50} when examined at either 12 or 19 h post inoculation (Table 4). They were, however, increased when incubated with 250 pg/ml endotoxin and

Table 2. Levels of TNF as determined by ELISA assay in supernatants harvested from human peripheral blood leucocytes after 2, 4, 12, 16, 20 or 28 h total incubation following treatment with influenza virus and endotoxin (LPS) for various periods

	Levels of TNF (ng/ml) after incubation for:					
Treatment	2 h	4 h	12 h	16 h	20 h	28 h
Cells alone*				0 (0)	0 (0)	0 (0)
Cells + LPS†	0.82 (0.03)¶	1.32 (0.07)	1.57 (0.33)‖	0.94 (0.25)**	0.75 (0.19)‖	0.55 (0.26)
Cells + virus‡	0 (0)	1.80 (0.13)	3.65 (0.76)	2.42 (0.38)	2.29 (0.34)	1.50 (0.34)
Cells + virus + LPS (1 h)§	0 (0)	1.33 (0.37)	3.53 (1.27)	3.26 (0.90)	3.65 (0.96)	2.06 (1.03)
Cells + virus + LPS (2 h)§			5.96 (0.77)‖	4.20 (0.17)**	6.42 (1.10)‖	3.49 (0.74)
Cells + virus + LPS (4 h)§			3.07 (0.02)‖	1.80 (0.01)	3.99 (0.93)	1.26 (0.26)

* No virus or LPS added.

† LPS (10 ng/ml) was added 1 h after initial cultivation of cells and the cells incubated for a further 1, 3, 11, 15, 19 or 27 h.

‡ Virus (100 EID_{50}) was incubated with cells for 1 h prior to removal and the cells incubated for a further 1, 3, 11, 15, 19 or 27 h.

§ Virus (100 EID_{50}) was incubated with cells for 1 h at which time LPS (10 ng/ml) was added (1 h), or LPS was added 1 h after removal of virus (2 h) or 3 h after removal of virus (4 h). Cells were incubated for further time periods (as shown).

¶ Standard error of the mean in parentheses.

‖ Significantly different at $P < 0.05$.

** Significantly different at $P < 0.01$.

Table 3. Levels of IL-6 as determined by ELISA assay in supernatants harvested from human peripheral blood leucocytes after 2, 4, 12, 16, 20 or 28 h total incubation following treatment with influenza virus and endotoxin (LPS) for various periods

	Levels of IL-6 (ng/ml) after incubation for:					
Treatment	2 h	4 h	12 h	16 h	20 h	28 h
Cells alone*				0 (0)	0 (0)	0.66 (0.65)
Cells + LPS†	2.52 (2.23)¶	22.6 (1.9)	136.72 (16.81)	16.63 (2.26)‖	226.19 (29.35)	255.03 (63.3)
Cells + virus‡	2.31 (0.1)	23.6 (2.92)	152.76 (9.5)	146.80 (20.09)	201.55 (1.23)	225.87 (23.29)
Cells + virus + LPS(1 h)§	0 (0)	28.3 (7.6)	186.48 (26.72)	173.39 (16.96)	233.80 (21.73)	257.64 (31.85)
Cells + virus + LPS (2 h)§			178.15 (5.87)	192.96 (9.68)‖	267.19 (5.47)	282.46 (2.70)
Cells + virus + LPS (4 h)§			131.57 (5.42)	75.00 (60.12)	186.30 (37.2)	211.27 (8.48)

* No virus or LPS added.

† LPS (10 ng/ml) was added 1 h after initial cultivation of cells and the cells incubated for a further 1, 3, 11, 15, 19 or 27 h.

‡ Virus (100 EID_{50}) was incubated with cells for 1 h prior to removal and the cells incubated for a further 1, 3, 11, 15, 19 or 27 h.

§ Virus (100 EID_{50}) was incubated with cells for 1 h at which time LPS (10 ng/ml) was added (1 h), or LPS was added 1 h after removal of virus (2 h) or 3 h after removal of virus (4 h). Cells were incubated for further time periods (as shown).

¶ Standard error of the mean in parentheses.

‖ Significantly different at $P < 0.01$.

combination of virus and endotoxin enhanced TNF-α levels compared with the corresponding dose of endotoxin alone; this enhancement over endotoxin or virus alone was statistically significant ($P < 0.05$) (Table 4).

Spontaneous levels of IL-1β were also low and not increased in cells incubated with 10 EID_{50} of virus (Table 4). They were increased about ninefold after treatment with 250 pg/ml of endotoxin but combination of virus and endotoxin did not enhance yields compared with endotoxin alone (Table 4).

As with TNF-α and IL-1β, the cells also spontaneously released IL-6 and the levels were increased by incubation for 1 h with 10 EID_{50} of virus or 250 pg/ml endotoxin but combination of virus and endotoxin did not significantly enhance the production of IL-6 compared with virus or endotoxin alone (Table 4).

Two further experiments (data not shown) also confirmed that 250 pg/ml of endotoxin and 10 EID_{50} of virus in combination did not significantly improve the enhancement seen with 10 ng/ml endotoxin and 100 EID_{50} virus.

Relation between bioassay titres and ELISA titres

Since it was possible that the enhanced yields of cytokines induced by endotoxin and virus combined did not reflect enhanced yields of biologically active cyto-

Table 4. Levels of TNF and IL-6 as determined by ELISA assay in supernatants harvested from human peripheral blood leucocytes after 12 or 19 h total incubation following treatment with influenza virus and endotoxin (LPS)

	TNF		IL-1		IL-6	
Treatment	12 h	19 h	12 h	19 h	12 h	19 h
Cells alone*	0.05 (0.04)¶	0.38 (0.14)	0.01 (0)	0.03 (0)	2.78 (0.3)	3.06 (0.08)
Cells + LPS†	0.81 (0.06)	0.69 (0.03)‖	0.32 (0)	0.28 (0.03)	12.87 (2.2)	13.82 (2.04)
Cells + virus‡	0.03 (0.03)	0.28 (0)‖	0.03 (0.02)	0.03 (0.01)	3.48 (0.7)	5.70 (0.36)
Cells + virus + LPS(1H)§	1.27 (0.29)	1.58 (0.17)‖	0.26 (0.06)	0.21(0)	7.65 (1.4)	10.19 (0.62)

* No virus or LPS added.

† LPS (250 pg/ml) was added 1 h after initial cultivation of cells and the cells incubated for a further 11 or 18 h.

‡ Virus (100 EID_{50}) was incubated with cells for 1 h prior to removal and the cells incubated for a further 11 or 18 h.

§ Virus (100 EID_{50}) was incubated with cells for 1 h at which time LPS (250 pg/ml) was added (1 h). Cells were incubated for a further 10 or 17 h.

¶ Standard error of the mean in parentheses.

‖ Significantly different at $P < 0.05$.

Table 5. Levels of TNF and IL-6 as determined by bioassay in supernatants from human peripheral blood leucocytes at various times following incubation with influenza virus and endotoxin (LPS)

Treatment	TNF (pg/ml)	IL-6 (U/ml)
Cells alone*	<50	29 (3)
Cells + LPS†	<50	6467 (148)
Cells + virus‡	<50	2400 (1437)
Cells + virus + LPS (1 h)§	150 (50)¶	6082(0)
Cells + virus + LPS (2 h)§	125 (75)	4712 (1370)
Cells + virus + LPS (4 h)§	75 (25)	8365 (769)

* No virus or LPS added.

† LPS (10 ng/ml) was added 1 h after initial cultivation of cells and the cells further incubated for 18 h.

‡ Virus (100 EID_{50}) was incubated with cells for 1 h prior to removal and the cells further incubated for 18 h.

§ Virus (100 EID_{50}) was incubated with cells for 1 h at which time LPS (10 ng/ml) was added (1 h), or LPS was added 1 h after removal of virus (2 h) or 3 h after removal of virus (4 h). Cells were incubated for a total of 19 h.

¶ Standard error of the mean in parentheses.

kine, a further experiment was performed in which levels of IL-6 and TNF were compared using the bioassay. Titres of TNF were considerably lower when determined by the bioassay than when determined by ELISA but the general pattern was maintained. No spontaneous release of TNF from cells from this donor was detectable, neither was any detected after stimulation with virus or endotoxin alone (Table 5). However, when virus and endotoxin were combined TNF could be detected at significant levels. The pattern of results for IL-6 by the bioassay agreed with that observed previously with ELISA in showing little enhancement in cytokine levels for virus and endotoxin in combination compared with either endotoxin or virus alone (Table 5).

Discussion

These studies confirm previous studies which have shown induction of IL-1, IL-6 and TNF by influenza virus or its proteins from monocytes or macrophages *in vivo* and *in vitro* (Jakeman *et al.* 1991; Lonnemann *et al.* 1989; Nain *et al.* 1990; Hinder *et al.* 1991; Vacheron *et al.* 1990). Also, it has been shown that endotoxin induces IL-1 (Mizel *et al.* 1978), IL-6 (Roth *et al.* 1992) and TNF (Hinder *et al.* 1991; Nain *et al.* 1990). Similarly, the potentiating effect of TNF-α production by influenza virus and bacterial endotoxin has been noted previously (Hinder *et al.* 1991; Nain *et al.* 1990) as has the potentiating effect of influenza virus and endotoxin on the release of another inflammatory mediator, histamine (Clementsen *et al.* 1988). However, as far as we are aware the potentiation of IL-1β and IL-6 production by influenza virus and endotoxin has not been examined previously.

The magnitude of the TNF-α enhancement by incubation with influenza virus and endotoxin in comparison with endotoxin alone was not as large (about threefold) as that reported previously by Nain and colleagues (about ninefold). This may be due to several differences in the experimental protocol. In our experiments, whole peripheral blood leucocyte preparations consisting of lymphocytes, neutrophils, eosinophils and basophils as well as monocytes (Tinsley *et al.* 1987) were used in suspension within 4–5 h of removal from the donor whereas adherent mononuclear cells from human peripheral blood leucocytes were used after 2–4 days in culture in the studies of Nain *et al.* (1990). We chose to do our experiments with a mixed population of cells since inflammatory cells induced in response to influenza virus are similarly mixed (Toms *et al.* 1977). In addition, culture of mononuclear cells *in vitro* for several days prior to performing the experiments may well alter the ability of such cells to produce cytokines. This is supported by the observation that, in contrast to our previous studies (Sweet *et al.* 1985) and that of others (Roberts & Horan 1985; Rodgers & Mims 1982) where only abortive cycles of replication occurred, their monocyte cultures supported productive replication of influenza virus. A further difference was the source of endotoxin which, in our experiments, was *E. coli* 055:B5 while in those of Nain *et al.* (1990) was *E. coli* 0127:B8; however, previous studies have shown 055:B5 endotoxin to be ~2–2.5-fold more active at inducing IL-1 activity than the endotoxin from the 0127:B5 strain (Newton 1986). Although the enhancement of TNF-α and IL-β yields by virus and endotoxin in combination were small they may be important *in vivo*. Similarly, although no enhancement of IL-6 levels occurred when cells were stimulated with both endotoxin and virus, the levels induced by either alone were often tenfold higher than those of TNF-α and IL-1β which again may be relevant *in vivo*.

What relevance might these observations have to cot deaths? Previously we have shown that endotoxin was lethal for 5-day-old ferrets and that its toxicity was enhanced in animals infected at 1 day old with influenza virus by up to 84-fold (Jakeman *et al.* 1991). No increased viral replication occurred in any tissue and the neonates died suddenly without clinical symptoms as in human babies dying from SIDS. Pathological examinations showed inflammation in the upper respiratory tract, lung oedema and collapse, and early bronchopneumonia in the toxin and influenza virus-treated animals but not in those treated with toxin or virus alone. Thus, bacterial toxins could play a role in SIDS, this being more likely with a concomitant influenza virus infection. The current experiments suggest that both influenza virus and endotoxin can stimulate the production of inflammatory cytokines and that the amounts of such cytokines may be enhanced by concomitant inoculation with virus and endotoxin. In this regard, it is interesting that elevated IL-6 levels have been demonstrated recently in cerebrospinal fluid obtained from SIDS victims (Vege *et al.* 1992). It may thus be instructive to examine cytokine levels in relevant tissues from SIDS victims to see whether such cytokines are produced in inappropriate amounts and we hope to examine this using PCR and in-situ hybridization.

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