Organoselenides as potential immunostimulants and inducers of interferon gamma and other cytokines in human peripheral blood leukocytes

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Summary. A number of organoselenium compounds have been described as anti-inflammatory, antioxidant, glutathione peroxidase-like agents and inhibitors of prostaglandin synthesis. Here we report that bis [2-(N-phenyl-carboxamido)]phenyl diselenide, 2-phenyl-1,2-benzisoselenazol-3(2H)-one (Ebselen) and related compounds are inducers of interferon gamma (IFN-γ) and tumor necrosis factor (TNF) in human peripheral blood leukocytes. The IFN and TNF response was rapid, occurring within 20 h, and high – up to 1000 and 2000 units ml^{-1} – and was clearly related to the dosage and the structure of the compounds. The action of the compounds and phytohemagglutinin was synergistic. The IFN gamma and TNF production was reduced after removing adherent cells. Although the mode of action of the compounds is not known, they appear to interact directly or indirectly with both adherent and non-adherent leukocytes, and stimulate the synthesis of a set of different cytokines including factors controlling the cell proliferation. Therefore, organoselenides may be regarded as the biological response modifiers.

Key words. Organoselenium compounds; Ebselen; interferon gamma; tumor necrosis factor; human peripheral blood leukocytes; interferon inducers; phytohemagglutinin; antiviral agents.

Interferon-γ (IFN-γ) is a product of lymphocytes and plays a crucial role in various immune fractions. Relatively few substances are known to induce IFN-γ and none of them is a clinically useful drug. The IFN-γ inducers include mitogenic lectins (phytohemagglutinin [PHA], Concanavalin A); bacterial mitogens (staphylococcal enterotoxin A and protein A, streptolysin O, lipopolysaccharide – LPS); antigens, antibodies (anti-lymphocyte serum, Mab OKT-3), and low molecular weight compounds (phorbol esters, calcium ionophore)[1,2].

It has also been suggested that modification of the cell surface by mild oxidation with sodium periodate or hydrogen peroxide, or by treatment with galactose oxidase or $ZnCl_2$, induces polyclonal proliferation of lymphocytes and release of IFN-γ and interleukin 2 (IL-2)[3,4].

Almost all of the IFN-γ inducers may also stimulate other cytokines including tumor necrosis factor (TNF) and interleukins 1 or 2. The most common results of their direct or indirect actions are stimulation or inhibition of proliferation of the lymphoid cells and generation of cytotoxic reactions[1–4].

We have described the synthesis of a number of organoselenium compounds including organoseleninic acids and organic diselenides which could be biologically active[5,6]. Some of the selenoorganic structures have been described by others as anti-inflammatory or anti-oxidant, or as eicosanoid inhibitors or glutathione peroxidase-like agents[7–9]. At present Ebselen (PZ-51) is undergoing clinical trials for rheumatoid arthritis and other diseases[9,10]. The in vivo toxicity of Ebselen is low, probably because the selenium in its structure is not bioavailable[8].

Here we report that diselenides and related compounds are potent inducers of IFN-γ and TNF in human peripheral blood leukocytes (PBL).

Materials and methods

PBL from healthy blood donors were used. The pooled leukocytes from two buffy coats were stored overnight at 4 °C and the red cells were lysed by two cycles of ammonium chloride treatment[13]. PBL were suspended in cold RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), penicillin 100 IU ml^{-1} and streptomycin 100 µg ml^{-1}. The cells were kept at 0–4 °C to avoid the adherence of cells to the glass.

The selenoorganic compounds were synthesized as described[5,6]. The stock solutions of the chemically pure and endotoxin-free, crystalline compounds (20 mg ml^{-1}) were prepared in dimethyl sulfoxide (DMSO). Because the compounds were practically water-insoluble the working fine suspensions of crystals were made in the culture medium.

Cytotoxicity of the drugs was determined in human lung carcinoma cell line A549 by viable cell staining of 24- and 48-h-old monolayers with 0.015% solution of neutral red (Serva) in Dulbecco's modified Eagle medium supplemented with 10% FCS[16].

Various concentrations of the compounds were added to the PBL suspensions ($8-10\times10^6$ cells ml^{-1}) and the cultures were incubated in an atmosphere of 5% CO_2 in air at 37 °C for 24 h. The cultures were then centrifuged and supernatants collected for IFN and TNF assays. The antiviral activity of IFN was determined in a cytopathic effect inhibition assay performed in monolayers of A549 cells in 96-well plastic microplates. The challenge was

encephalomyocarditis virus (EMCV)[11]. A similar assay was used by the WHO Committee on Interferon Standardization[12]. Recombinant human IFN-γ (Genentech Inc., San Francisco, CA, spec. Act. 2×10^8 units mg^{-1}, provided by M. C. Shepard) and natural human leukocyte IFN-α (3×10^6 I.U. ml^{-1}, obtained from K. Cantell, Helsinki, Finland) were used as reference.

The cytotoxic activity of TNF was measured in L 929 cells[14]. The samples and actinomycin D solution were added to monolayer cultures of the cells. After incubation for 20 h at 37 °C the cultures were stained with crystal violet and toxic effect was determined. The amount causing 50% destruction of the cell cultures was defined as one unit of TNF activity. Comparison with a standard preparation of TNF-α (Genentech Inc., USA; a gift of Dr M. C. Shepard) showed that 1 unit in our assays was equal to $4-8 \times 10^{-12}$ M TNF.

Results and discussion

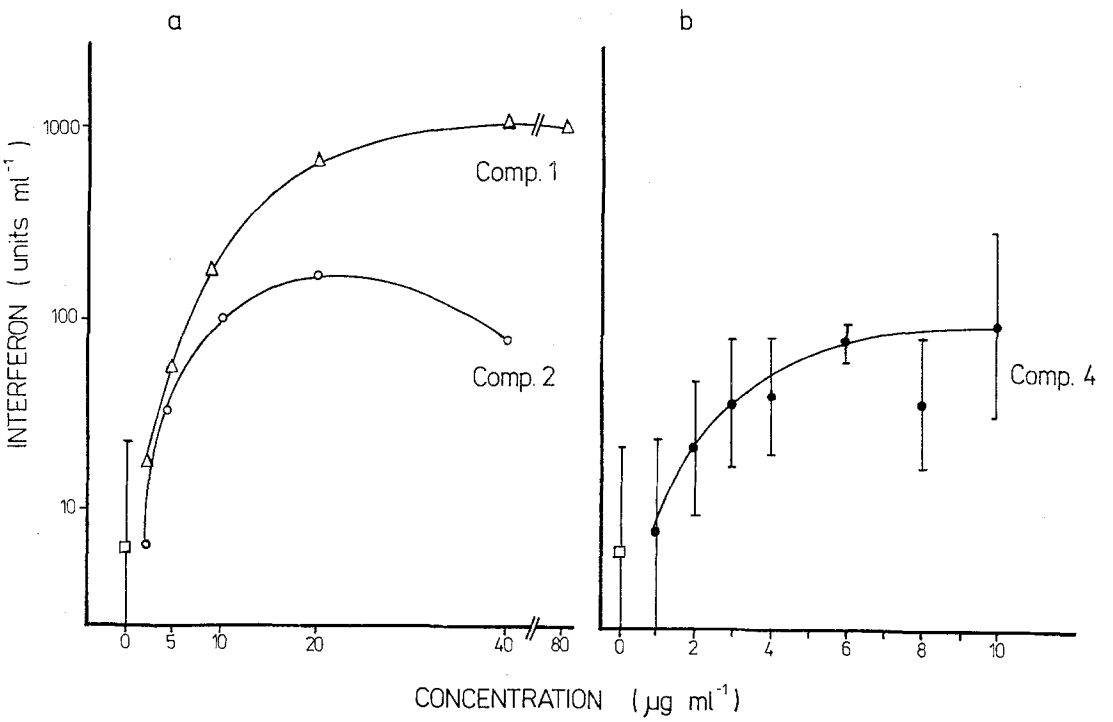
Treatment of PBL with various concentrations of compounds **1–3** resulted in rapid and high IFN synthesis. Compound **4** had weaker IFN-inducing activity. The IFN response, as measured by a bioassay[11,12], was clearly related to the dosage and the structure of the compounds (table 1, fig. a, b). The IFN-inducing activity of the compounds was apparently not correlated with their cytotoxicity because the most active compounds, **1** and **3**, showed the lowest toxicity (table 1). Unexpectedly, compound **1**; bis [2-(N-phenylcarboxamido)]phenyl diselenide, recognized by others as a metabolite of compound **2**; 2-phenyl-1,2-benzoisoselenazol-3 (2H)-one (Ebselen)[8], was found to be the most potent IFN inducer. This suggests that the observed activity of Ebselen is due to diselenide 1 being formed in the PBL culture by a metabolic pathway.

The selenoorganic compounds were also inducers of another cytokine, TNF. The most potent compounds in this respect were **1**, **2** and **3**, whereas compound **4** was the

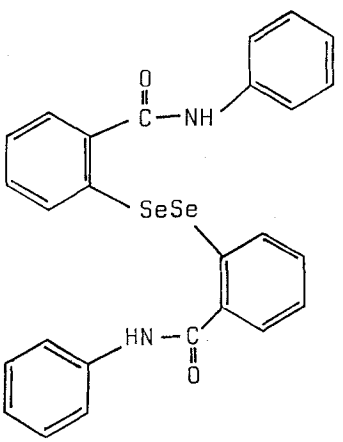
Table 1. Structure-activity relationship of four selected selenoorganic compounds as IFN and TNF inducers in PBL cultures

Compound No.	Structure	Minimum cytotoxic concentration ($\mu g\ ml^{-1}$)	Maximum IFN response (units ml^{-1})	Maximum TNF response (units ml^{-1})
1		170	1000	2000
2		50	300	750
3		80	700	500
4		20	100	100

1 Bis [2-(N-phenylcarboxamido)] phenyl diselenide; **2** 2-Phenyl-1,2-benzoisoselenazol-3(2H)-one (Ebselen, PZ 51); **3** Bis [2-(N-(2-pyridyl)carboxamido)] phenyl diselenide; **4** Bis (2-pyridyl) diselenide.



Dose response of PBL to the IFN-inducing effect of compounds **1** and **2**(a) and **4**(b). Curves in (a) show mean values of two typical experiments, whereas in (b) they are the means of 13 experiments. □ Shows the spontaneous production of IFN-γ in PBLs without any exogenous inducers. For further explanations see table 1.



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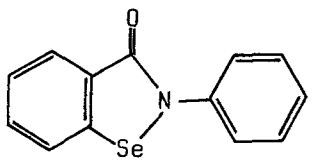
weakest (table 1). We have compared the IFN-inducing activities of PHA acting by itself or in combination with the drugs. The combination showed a clear synergistic effect (table 2).

In contrast, there was no synergy between LPS from *E. coli* (0111: B4, Difco Labs, used at a concentration of 10 μg ml^{-1}) and compound **1** (10 μg ml^{-1}). The two compounds, used alone or in combination, induced 1.89 ± 0.11; 1.88 ± 0.56 and 1.5 ± 0.50 IFN-γ units ml^{-1}, respectively.

IFN and TNF production in PBL cultures treated with compound **1** or compound **2** was reduced approximately 4-fold after removal of the adherent cells by adsorption to a plastic plate for 2.5 h at 37 °C (data not shown).



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Under suitable experimental conditions all major acidic non-steroidal anti-inflammatory drugs share the property of inhibiting the multiplication of various viruses in cell cultures[15,16]. We examined the organoselenium compounds for their antiviral activity against two viruses which are used as a challenge in IFN assays: EMCV and vesicular stomatitis virus (VSV). We have found that at noncytotoxic concentrations compounds **1** and **2** inhibited viral cytopathogenicity and replication (table 3). However, the selectivity indices of the compounds were relatively low. For example, in the A549 cells-EMCV system, the ratios of the 50% cytotoxic dose to the 50% effective dose, based on the cytopathic effect assay, were 4.2 and 1.7 for compounds **1** and **2**, respectively. The removal of the compounds from the culture media abrogated their antiviral action.

The compounds **1**–**4** at concentrations of 10–30 μg ml^{-1} had no effect on the end-point of IFN-α or IFN-γ titration in the monolayer cultures of A549 cells when the media with the drugs were removed from the cultures before challenge with EMCV. We found that the compounds **1** and **2** did not induce IFN in human embryonic fibroblasts aged in vitro for 14 days.

Polyclonal sheep antibodies neutralizing Hu IFN-α or Hu IFN-γ (provided by K. Cantell, Helsinki, Finland) were used to identify the type of IFN induced in PBL cultures by the organoselenium compounds. Treatment with the anti-IFN-γ serum diluted 1:200 resulted in almost complete reduction of IFN induced by compounds **1**–**4**. In contrast, anti-Hu IFN-α serum only neutralized this IFN very weakly. Therefore, a major part of the IFN induced by the selenoorganic compounds was IFN-γ.

Table 2. Synergism in IFN-γ induction by selenoorganic compounds and PHA in PBL

Compound (μg ml^{-1})	IFN titer (log units ml^{-1}) Without PHA Actual	With PHA Actual*	Expected +	Fold potentiation ++
Diselenide **1** 10	1.88 ± 0.56	3.11 ± 0.39	2.29	6.61
2 Ebselen 10	2.09 ± 0.57	2.81 ± 0.58	2.39	2.63
Diselenide **3** 20	2.16 ± 0.23	3.33 ± 0.47	2.42	8.13
None	0.43 ± 0.47	2.08 ± 0.65	–	–

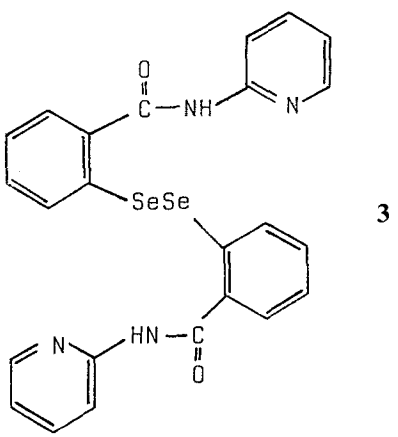
The PBL cultures contained 1.0×10^7 cells ml^{-1}. PHA refers to Leucoagglutinin (Pharmacia, Sweden), used at a concentration of 5–10 μg ml^{-1}. Incubation was carried out for 24 h at 37 °C. The doses of the organoselenium compounds used in the experiments were suboptimal for IFN-γ induction. * Actual IFN titers were determined as described in text. Mean ± standard deviation. + Expected IFN titers were determined by adding the actual titers of IFN induced by the compounds **1**, **2** or **3** and by PHA. ++ The potentiation factor was calculated by dividing the actual IFN titer by the expected IFN titer.

Table 3. Antiviral activity of two selected selenoorganic compounds in A549 cells

Virus	Assay	Minimum inhibitory concentration (μg ml^{-1}) of compound **1**	**2**
VSV	PFU*	30	20
EMCV	CPE^+	40	30
VSV	CPE^+	30	10

Required to inhibit: * virus plaque formation by approximately 90% or $^+$ virus induced cytopathogenicity by 50% during incubation at 37 °C for 24 h.

The drug concentrations used were not cytotoxic (see table 1). The multiplicity of infection was approximately 10 PFU/cell for VSV or EMCV.



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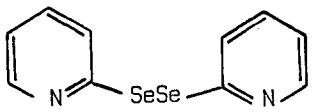

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However, we cannot exclude that a very small amount of IFN-α/β was present in the preparations.

In the 3–4-day-old cultures of PBL (10^6 cells ml^{-1}) in RPMI 1640 medium supplemented with 10% fetal calf serum and compounds **1** or **2** (10–90 μg ml^{-1}), stimulation of the incorporation of ^{3}H-thymidine was observed. This was evident in the presence or absence of PHA (1–5 μg ml^{-1}). The observations suggest that the selenoorganic compounds may also induce synthesis of mitogenic interleukins or other cytokines (data not shown). It can be concluded that organic diselenides and Ebselen are potent IFN-γ and TNF inducers in human PBLs but they do not stimulate IFN release in human fibroblasts. Ebselen may act as an IFN inducer after conversion to diselenide. Although the mode of action of the compounds is not known it appears to involve both adherent and non-adherent leukocytes. Because the selenoorganic compounds are undergoing clinical trials as anti-inflammatory drugs their novel IFN-γ and TNF-inducing activity and potential immunostimulating action deserve special attention.

It is worth pointing out that IFN-γ has been successfully used for treating patients with rheumatoid arthritis [17].

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Microtubule inhibitors block the morphological changes induced in *Drosophila* blood cells by a parasitoid wasp factor

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Summary. The shape change of *Drosophila melanogaster* blood cells (lamellocytes) from discoidal to bipolar that is caused by a factor from the female parasitoid *Leptopilina heterotoma* is blocked by the tubulin inhibitors vinblastine and vincristine in vitro. The actin inhibitor, cytochalasin B, causes arborization of *Drosophila* lamellocytes and acts synergistically with the wasp factor to alter lamellocyte morphology. Lamellocyte arborization induced by cytochalasin B is blocked by simultaneous treatment with vinblastine. These observations indicate that the changes in lamellocyte shape induced by both the wasp factor and cytochalasin B require microtubule assembly.
Key words. Drosophila melanogaster; Leptopilina heterotoma; parasitoid; blood cells; vinblastine; vincristine; cytochalasin B; cytoskeleton.

The ability of *Drosophila melanogaster* blood cells to encapsulate foreign objects is destroyed by the parasitoid *Leptopilina heterotoma* [1]. The female wasp injects a factor, named lamellolysin, along with its eggs that selectively incapacitates the capsule-forming blood cells of *Drosophila*. The destruction of these hemocytes, lamellocytes [2], is an orderly process in which the thin, discoidal cells become bipolar and shed their elongating cytoplasmic ends. The morphologically-altered lamellocytes are no longer adhesive so they are unable to layer around the parasitoid eggs to contain them and the eggs develop undisturbed in the host hemocoel.