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Conference Presentation

Biological activity of interferon gamma and lipopolysaccharide on the nitric oxide production in C6 astroglioma cells and some unexpected effects of potentization

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Abstract

Background: A proinflammatory environment is a hallmark of several neurodegenerative diseases where astrocyte involvement is also well established. Astrocytes and microglia in central nervous system are mainly involved in the release of cytokines, oxygen free radicals and nitric oxide (NO). Several studies on C6 astroglioma cells, a widely used in vitro model for these events, demonstrated that co-stimulation of this cell line with bacterial lipopolysaccharide (LPS) and interferon gamma (IFN-γ) induces a synergistic nitric oxide synthase (iNOS) expression.[1] In our laboratory we are using this versatile cell model in order to carefully investigate dose-response effects of various putative agonists or inhibitors and to assess the possible changes provoked in those agents by different procedures of dilution and succussion (agitation or shaking). Succussion is the physical basis of potentization or dynamization according to the homeopathic terminology.

Materials and methods: C6 rat astroglioma cells were cultivated in DMEM F12 (AUROGENE) cultured media supplemented with 10% FBS (LONZA) and 100 U/ml penicillin/streptomycin. This complete medium was replaced every two days. When the cultures became 70% confluent the cells were detached from the flask surfaces with trypsin 5 mg/l (Cambrex) in PBS and planted in black 96 multi well plate (Perkin-Elmer). $5x10^4$ cells per well were plated in DMEM F12 supplemented with 2% FBS, 100 U/ml penicillin/streptomycin and after 24 h, treated with different concentration of succussed or not succussed LPS from E. coli ORI 26:B6 (Sigma-Aldrich) and rat recombinant IFN-γ (R&D systems). Negative controls, done with the same solvent without IFN-γ but with LPS, were present in each experiment. The solutions were prepared starting from stock solutions of 1 mg/ml (LPS in distilled sterile-filtered water) and of 0.9 mg/ml (IFN-γ) serially diluted 10x, until the indicated doses, in distilled sterile-filtered water (Sigma-Aldrich) in a sterile glass test tube and, where indicated, strongly succussed in a Dyna-A mechanical shaker delivering 20 strokes/second with the hike of 11 mm. The effects of cell treatments were evaluated testing the NO production and secretion in the cultured media (nmoles nitrite/$5x10^4$ cells) after 24 h using Griess reagent. In a first analysis C6 cells were treated for 24 h with the indicated LPS dilutions (without and with succussion for 7.5 sec, 150 strokes) in the presence of 100 ng/ml IFN-γ. Then NO synthesis was

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evaluated for 24 h with the indicated IFN-γ dilutions (without and with succussion for 7.5 sec/150 strokes) in the presence of 10 ng/ml LPS. Finally we also analyzed the succussion effects treating cells with LPS 10 ng/ml + IFN-γ dilutions not succussed, succussed 7.5 sec (150 strokes) and succussed 7.5 sec three times (total 450 strokes). We tested also several preparative methods of IFN-γ dilutions: a) manual succussion (10 times manual shaking on an hard but elastic surface, e.g. a large book), b) 7.5 sec succussion with Dyna-A mechanical shaker, c) 8 sec vortexing with bench top vortex, d) gentle mixing by slow reversal of test tube by hand three times.

Results: Figure 1 shows dose-effect studies of LPS and IFN-γ. Preliminary experiments showed that NO release was triggered in a synergistic way by the two factors, i.e. only when suitable concentrations of LPS and IFN-γ were present in the colture media. No production of NO was detected in resting cells or in cells treated with LPS or IFN-γ alone. This kind of synergism is very interesting, considering that LPS is the major endotoxin of G-bacteria and that its possible leakage from gastrointestinal tract into lymph or bloodstream, with consequent presence even in small amounts in tissues affected by immunopathologic processes (where IFN-γ is also present), can trigger the release of huge amount of toxic nitrogen free radicals.

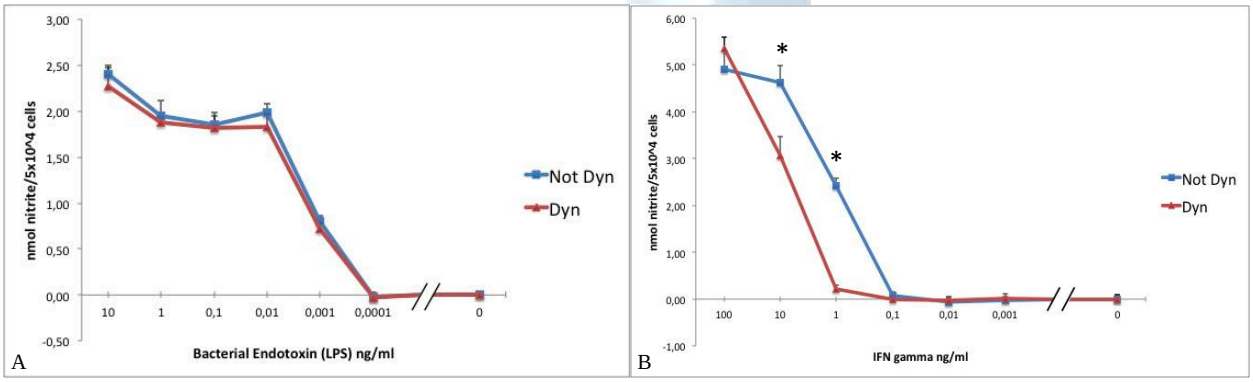

Figure 1: NO production by C6 cells treated with decreasing doses (increasing decimal dilutions) of LPS in the presence of 100 ng/ml IFN-γ (fig. 1A) and of IFN-γ in the presence of 10 ng/ml LPS (fig. 1B). The two agents were prepared by serial dilutions in water followed by gentle mixing (blue lines) or by serial dilutions in water followed by succussion 7.5 sec with Dyna-A mechanical shaker (red). * T-test $p<0.001$

In our experimental setting, mechanical succussion didn't affect the LPS activity (Fig. 1A) while with IFN-γ the dilution-effect curve of succussed solutions shifted to the left by about one order of magnitude (Fig. 1B). This indicates that the capability to induce NO synthesis was markedly reduced by succussion. Moreover, NO amount released in C6 treated cultured media decreased in response to increased number of succussion cycles (three times in our experimental method) (fig. 2A).

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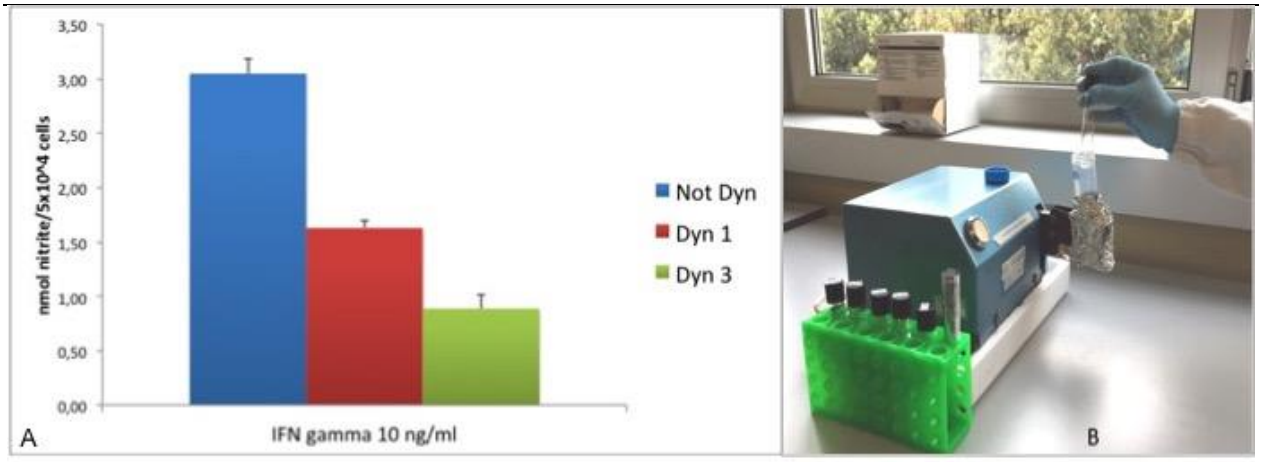

Figure 2: NO production in C6 cells treated with LPS 10 ng/ml plus IFN-γ 10 ng/ml not succussed or succussed one and three time. (Not Dyn = not succussed, Dyn 1 = succussed one time, Dyn 3 = succussed three times) (fig. 2A). The potentizer Dyna-A used in all experiments (fig. 2B).

Following these unexpected findings, we decided also to test the effects on nitric oxide production of several preparative methods of IFN-γ dilutions: manual succussion, succussion with Dina-A and vortex (Fig. 3A). All these treatments induced a decrease in the nitric oxide production and the main effective one was the manual succussion (Fig. 3B). This phenomenon was unexpected and paradoxical because, according to a simplified view of traditional homeopathic pharmacopoeia, the succussion should increase the pharmacologic power of drugs.

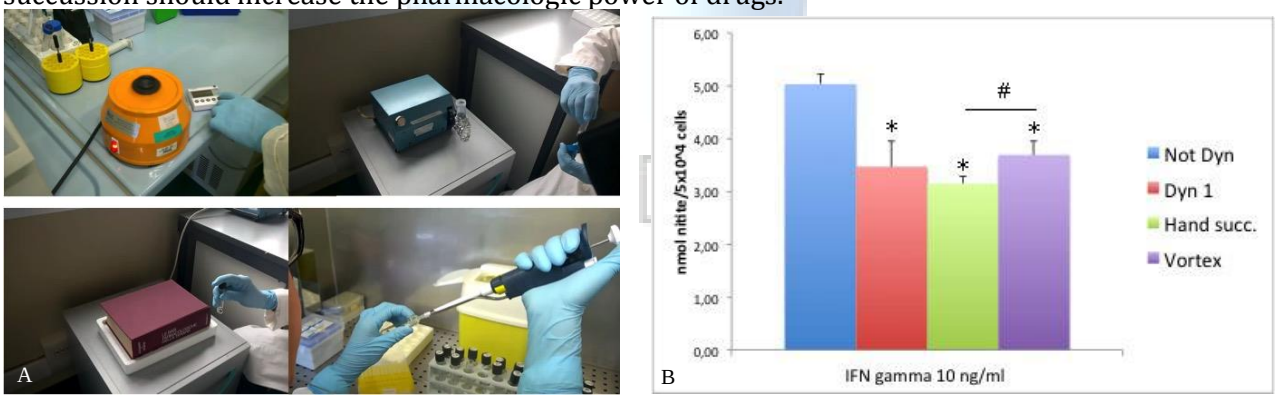

Figure 3. Effects of IFN-γ dilution and succussion. 3A: Clockwise from left to right: vortex, succussion with Dyna-A mechanical shaker, gentle mixing of sample not succussed, and hand succussion (for details see materials and methods). 3B: NO production in C6 cells treated with 10 ng/ml LPS plus IFN-γ diluted to the dose of 10 ng/ml with different procedures: not succussed or succussed with mechanical shaker Dyna-A or vortex, or hand succussed. (Not Dyn = not succussed, Dyn 1 = succussed one time, hand succ.= hand succussion, and vortex). * T-test $p<0{,}001$ vs Not Dyn and # T-test $p<0.01$ vortex vs hand succussion.

Discussion: Even if we did not explore higher dilutions/ potentized remedies in the homeopathic range (and this is a limit of our approach that prompts for further investigations), our results rule out the naïve hypothesis that dilution plus succussion – in the low-dose molecular range - increase

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"per se" the pharmacological power of a cytokine. On the other hand this succussion-dependent change could affect different substances in different ways and may have importance for the interpretation of the "simile" at the molecular level. We have recently pointed out how minor changes of protein structure could dramatically affect ligand-receptor interactions and thus the outcome of intercellular signaling, since different configurations of the same substance could have either "agonist" or "inverse agonist" effects[2] The succussion-dependent IFN-γ inactivation may have a physiologic role assuming that this important cytokine is "succussed" in vivo by circulatory system, attenuating the cytotoxic effects (like nitric oxide overproduction) of this compound secreted in high amount in inflammation diseases. The hypothesis that succussion could enhance – instead of diminishing - the power of other compounds can't be excluded from our data and could be also verified using other natural substances in this cellular model. We are currently investigating the mechanism behind this loss of activity of IFN-γ. A trivial explanation like absorption on glass walls of tube seems unlikely because this would have occurred also in non-succussed solutions due to rapid diffusion kinetics of protein in solution. A more probable explanation could be a denaturation of the homodimer molecular structure of IFN-γ during succussion. For instance, the exposure to mechanical stress or heating could lead to conformational changes in the protein structure or irreversible chemical modifications[3]. In particular, it has been reported that oxygen tension and heat treatment have a specific impact on IFN-γ modification and its biological activity during filling and storage[4]. Since the stability and/or change of activity of key cytokines has important implications in pathology and immunology, the observed succussion effect on protein structure and related biological functions is worthy of further investigations.

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Authors declares there is no conflict of interest

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Keywords: interferon gamma, homeopathy, dynamization, potentization

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