

Application of two *in vitro* methods for the toxicity test of autogenous vaccines

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Abstract

According to the 3Rs principle (Replacement, Refinement, Reduction), this study aims to find alternative methods to evaluate the toxicity of autogenous vaccines. Currently in Italy the Istituti Zooprofilattici Sperimentali (I.Z.Z.SS.) must perform the *in vivo* toxicity test for each lot of autogenous vaccine produced as laid down in the Decree of 17 March 1994. This paper describes two *in vitro* methods for assessing the toxicity of autogenous vaccines. The first is the MTT test based on the metabolic reaction of tetrazolium salt in vital cells. The second method is the test for measurement of IL-1 β production by macrophages, obtained after *in vitro* differentiation from pig monocytes in peripheral blood mononuclear cells. The two tests were performed on different vaccine antigens dilution: 1:20, 1:100 and 1:500. The results show a positive tendency between the two methods pointing out the potential of these methodologies combined for the replacement of the current *in vivo* test.

Introduction

Autogenous vaccines are immunizing products prepared against pathogenic microorganisms isolated from sick animals

of one herd and only usable in the same farm. The Decree of 17 March 1994 in Italy regulates the production and control of autogenous veterinary vaccine. The legislation provides that the I.Z.Z.SS. are the only agencies authorized by the Ministry of Health for the production of veterinary autogenous and autologous vaccines. Moreover, as required by the law, the I.Z.Z.SS. must perform the *in vivo* toxicity test (mouse model) for each lot of vaccine produced.¹ According to the 3Rs principle, this study aims to find alternative methods to evaluate the vaccines toxicity replacing the animal model.

Materials and Methods

In this study, the MTT *in vitro* method was applied to evaluate the cytotoxicity of autogenous vaccine antigens. Moreover, an *in vitro* method was developed to measure the release of interleukin IL-1 β by porcine macrophages in order to evaluate the potential *in vitro* inflammatory response triggered by the vaccine. The antigens analysed in the tests were inactivated with 0.8% formalin for 24 hours at 37°C and washed with saline solution, before the addition of the adjuvant.

The first method used is the cytotoxicity test MTT, based on the metabolic reaction of tetrazolium salt in vital cells. The MTT test is one of the most widely used, in accordance with the UNI EN ISO 10993-5 standard, concerning the biological evaluation of medical devices, which allows evaluating the safety and toxic potential of products.² For the test, the L-929 cell line was used, seeded at the concentration of 1.0x10⁵ cells/mL in 96-well plates in MEM culture medium added with 10% of foetal bovine serum (FBS), and incubated at + 37°C \pm 1°C, 5% of CO₂. After an overnight incubation, the vaccine antigens were diluted and distributed 100 μ L/well. After 24 hours of incubation, the cell monolayer was treated with MTT salt and the intensity of the colorimetric reaction was correlated to cell viability.

The second method performed is a test for the assessment of IL-1 β production by porcine macrophages in response to vaccine antigens. Interleukin-1 β (IL-1 β) is a pro-inflammatory cytokine mainly produced by activated macrophages and monocytes. The precursor (pro IL-1 β) is located at cytoplasmic level and must be cleaved by caspase-1 to generate the mature activated form.³ Assessment of IL-1 β production by macrophages in response to vaccine anti-

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gens could help evaluate the safety and efficacy of the vaccine-induced immune response. In particular, macrophages were

obtained after *in vitro* differentiation from peripheral blood mononuclear cells (PBMC), using 10 ng/mL of Macrophage-Colony Stimulating Factor (M-CSF). Differentiated macrophages were reacted with the same antigens at different dilutions for 24 hours, followed by quantification of released IL-1 β by "Duo set ELISA for Porcine IL-1 β /IL-1F2" (R&D System).

Results

The results showed two crucial points to consider for the IL-1 β assessment *in vitro* test: i) the choice of the cell population, because macrophages with high basal reactivity tended to provide a *plateau* response; ii) the need to dilute the antigens because their high bacterial content caused an effect "endotoxin tolerance-like", that inhibits the release of IL-1 β as showed in Figure 1.⁴

Furthermore, the MTT test results showed that the samples are cytotoxic at lower dilutions, showing a cell viability \geq 70% from at least 1:8. The maximum dilution, to which a cytotoxic effect was so far detected, looks to be 1:128.

Moreover, to assess the correlation between the two methods, the results of the MTT test at the dilutions 1:16 were trans-

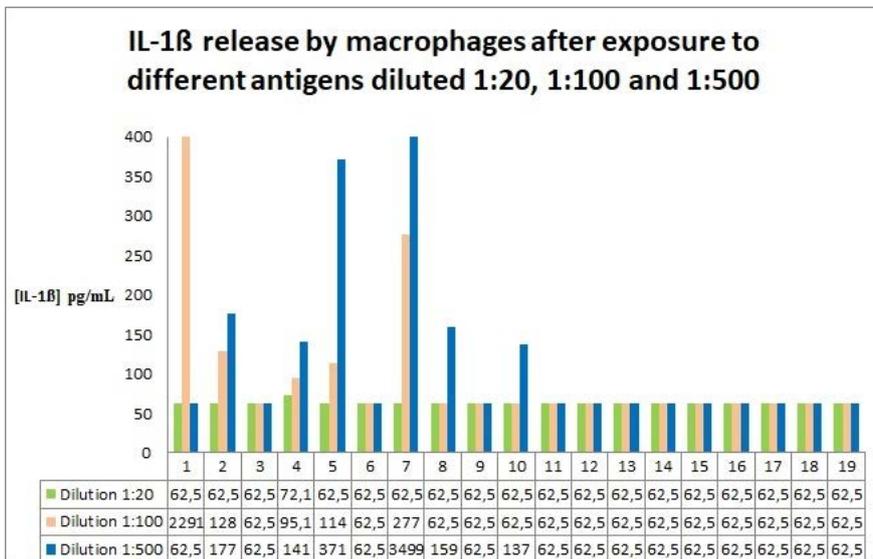


Figure 1. IL-1β release by macrophages after exposure to different antigens.

formed into a percentile value and were correlated via *GraphPad Prism* software with the concentrations of IL-1β detected at 1:500 dilutions of the antigens.

Conclusions

The statistical analysis, carried out using the Spearman coefficient, shows a positive tendency between the two methods (R=0.4955, P=0.03) pointing out the poten-

tial of these combined methodologies to examine different features of the immune/toxic response, that autogenous vaccine could trigger in the target animals.⁵

The study confirms the scientific value of the 3Rs and their applicative potentiality, especially when alternative *in vitro* methods are combined in integrated assay strategies to enhance, through multiple end-points, the effectiveness and predictive capacity of the models.

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