Use of bovine lymphocytes to assess the immunomodulatory effect of natural extracts

L. Lignitto[1], L. Da Dalt[2], S. Balzan[3], G. Gabai[2], S. Segato[1], R. Ricci[1], E. Novelli[3]

[1] Dipartimento di Scienze Animali. Università di Padova, Italy
[2] Dipartimento di Scienze Sperimentali Veterinarie. Università di Padova, Italy
[3] Dipartimento di Sanità Pubblica, Patologia Comparata e Igiene Veterinaria. Università di Padova, Italy

Corresponding author: Laura Lignitto. Dipartimento di Scienze Animali. Facoltà di Medicina Veterinaria, Università di Padova. Viale Dell'Università 16, 35020 Legnaro (PD), Italy - Tel. +39 049 8272777 - Fax +39 049 8272633 - Email: laura.lignitto@unipd.it

ABSTRACT: A simple and rapid method based on the *in vitro* bovine lymphocyte proliferation was developed to assess the immunomodulatory activity of natural compounds extracted from plant and foods. The proliferation of lymphocytes was measured by the MTT [3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] assay. This test was used to measure the immuno-enhancing activity of 2 plant extracts and 2 samples of fermented milk after 48 h of incubation. To evaluate the effect of the cow physiological phase on the assay response, the tests were repeated using lymphocytes taken from non-pregnant dry, pregnant dry and post partum cows. The assay was not affected by the cow physiological phase and the results obtained were comparable. Both plant extracts enhanced lymphocyte growth, in particular the extract 2 was slightly more potent than extract 1. The fermentation of milk with proteolytic starter cultures, such as FAIR E-63 and LA 2, can generate peptidic compounds from milk protein with potential immunostimulating activity. These samples tested in cell culture inhibited lymphocyte proliferation because they showed an acidic pH of 4.5. However, this bioassay was enough sensitive to detect biological activity of different compounds also at low concentrations.

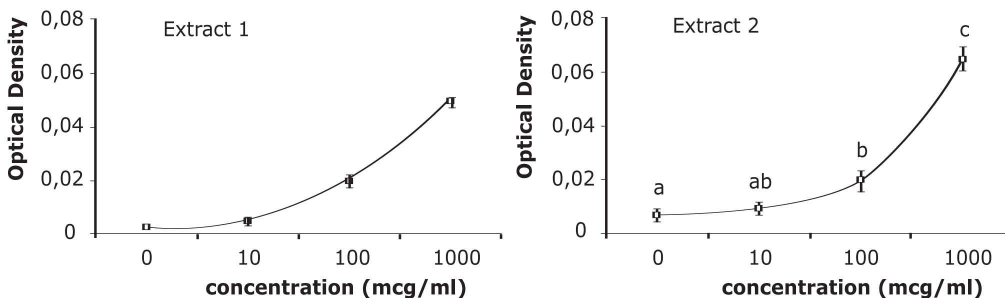
Key words: Bovine lymphocyte, Bioassay, Immunomodulation, Food components.

INTRODUCTION – There are many methods used to study immunomodulation of immune cells. Direct visual cell counting using a hemocytometer can be accurate but it is too time-consuming and laborious in screening applications. Alternative *in vitro* assays have been developed to assess cell proliferation such as incorporation of tritiated thymidine ([^{3}H]TdR) into the DNA of proliferating cells (Messele *et al.*, 2000). However, issues associated with the cost and safety of the use and disposal of radioactive materials has led to the search for viable nonradioactive alternatives to measure cell proliferation in cultured cells. The development of monoclonal antibodies directed against 5'-bromo-2'-deoxyuridine (BrdU), a chemical analog of thymidine, has led to design of methods to measure DNA syntesis in cultured cells by both immunocytochemistry (counting labelled nuclei) or by BrdU enzyme-linked immunosorbent assay (ELISA) in which the amount of BrdU incorporated into cultured cell in microplates is quantitated colorimetrically in a microplate reader (Magaud *et al.*, 1988). This approach allows relatively rapid quantification of DNA synthesis in a large number of samples. Several alternative methods have also been proposed, such as staining with crystal violet (Gillies *et al.*, 1986), staining of cell-associated protein (Tuszynsky and Murphy, 1990), measurement of intracellular enzyme activities or measurement of metabolic activities (cytokine production). In this report, we used the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (Hansen *et al.*, 1989). The yellow tetrazolium MTT is reduced by the action of dehydrogenase enzymes of metabolically active cells to an intracellular purple formazan that can be solubilized and quantified by spectrophotometric means.
Bovine lymphocytes are easily available and can be good candidates for bioassay development. Moreover, we are interested in studying the effects on the bovine immune system of compounds derived from animal food and immunomodulatory peptides encrypted in milk proteins, which can be released by bacteria present in the mammary gland.

MATERIAL AND METHODS – The immunomodulatory potentials of two vegetal extracts (named: extract 1; extract 2; INDENA, Milano, Italy) with proven immunostimulating activity in other bioassays and two samples of fermented milk were screened by the MTT assay on bovine lymphocytes taken at three different physiological phases: non-pregnant dry, pregnant dry and post partum cows. The dose response curves were calculated for the 4 compounds under investigations, and the effect of the physiological phase was studied by ANOVA (SPSS 14.0).
Bovine lymphocyte cultures. Blood (10 ml) was collected in heparinized tubes from 9 Italian Friesian pluriparous cows selected as follows: 3 non-pregnant dry, 3 pregnant dry (15±5 days before partum) and 3 lactating (19±6 days *post*-partum). Three ml of blood were diluted 2-fold with NaCl 0.9% and layered onto 3 ml of Lymphoprep™ (Sentinel, Milano, Italy). Lymphomonocytes were separated as a thin layer over the Lymphoprep by centrifugation at 800 x *g* for 25 minutes. Cells were washed twice with NaCl 0.9% and subsequently diluted in RPMI-1640 medium (Invitrogen, Milano, Italy) containing 10% newborn calf serum (Sigma, Milano, Italy), 2mM L-glutamine (Sigma, Milano, Italy), streptomycin (100 µg/ml, Invitrogen, Milano, Italy), penicillin (100 units/ml, Invitrogen, Milano, Italy) and 2 µg/ml concanavalin A (Sigma, Milano, Italy). Cells were incubated for 24 h at 37°C in an atmosphere containing 5% CO_2. Then, lymphocytes were separated from adherent monocytes and diluted in fresh medium without concanavalin A to be used for the assays. Lymphocytes were seeded in a 96 well plate at a concentration of 1.5 x 10^6 cells/ml. Lymphocytes were then treated with various concentrations of the compound under study (0 to 1000 µg/ml) and incubated for 48 h at 37°C.
Milk fermentation. Dry skimmed milk was rehydrated (10%) and autoclaved (121°C for 5 min). The prepared milk was inoculated with *Enterococcus faecalis* (FAIR E-63) and *Lactobacillus delb. bulgaricus* (LA 2) and incubated at 37°C and 44°C, respectively, for 24 h. Milk samples were prepared by centrifugation (7000 x *g*, for 15 min at 4°C; Beckman Counter, Fullerton, California). The supernatant was centrifugated again (20,000 x *g*, for 15 min at 4°C) and the new supernatant was filtered using Centricon Amicon Ultra 15 filters (cut off 5000 Da) by centrifugation (3200 x *g*, for 40 min at 15°C). The samples with peptidic fractions (< 5000 Da) were used in these experiments.
Lymphocytes proliferation. The effect of natural extracts on the lymphocytes viability was measured using the 3-(4, 5-di-methylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenases of the viable cells. At the end of 48 h incubation, 10 µl of MTT solution (5mg/ml, Sigma, Milano, Italy) was added to each well and incubated for 2 h at 37°C. The formazan was eluted into the medium by adding 50 µl of acidified Triton X-100 solution and shaking for 45 minutes. Bioassay response was quantified by reading the assorbance at 570 nm at the reference wavelength of 630 nm using a SpectraCount (PerkinElmer, Massachusetts, USA). The relative cell viability and proliferation was determined by the amount of MTT converted to the insoluble formazan salt.

RESULTS AND CONCLUSIONS – In the present study, we evaluated the immunomodulatory activity of two plant extracts and two samples of milk fermented by FAIR E-63 and LA 2 bacterial strains in bovine lymphocytes.

Figure 1. Dose-response curves obtained for the two vegetal extracts. Different letters indicates significantly different means (P<0.01; Waller-Duncan test, SPSS 14.0).



The immune cells were incubated with or without different concentrations of the extracts for 48 h. The activity was assessed by testing the effect on lymphocytes growth using a rapid test such as MTT assay. The assay was not affected by the cow physiological phase. Thus, results obtained using lymphocytes taken at different physiological phases were comparable.
The bioassay showed a high degree of variability at the lower concentrations of the compounds under test, in fact the coefficient of variability within the assay (CVw) was 21.6% while the coefficient of variability between the assays (CVb) was 80.4%, as measured at 10 µg/ml of added test compound. Although the high variability, the test was suitable to measure the immunostimulating activity of both plant extracts (figure 1). In our system, the extract 2 was slightly more potent than extract 1.

The bacterial strains FAIR E-63 and LA 2 were selected as starters as the showed high proteolitic activity (1.58 mg Tyr/5 ml and 0.50 mg Tyr/5ml, respectively). Thus, they may produce several oligopeptides and bioactive peptides from milk protein precursors. The low molecular weight fraction samples of milk fermented by FAIR E-63 and LA 2 did not stimulate lymphocytes proliferation. Rather, they showed an acidic pH of 4.5 and inhibited cell growth. Matrix characteristics must be carefully considered when this bioassay is used. At the moment, fermented milk samples are subjected to peptide precipitation and subsequent solubilization in culture medium before used in the assay.
According to the low value of CVw, the application of this bioassay is valuable to study biological activity of different compounds although it is not useful for quantification the biological activity of the same extract in different samples (CVb high). Thus, it will be necessary to determine the accuracy and the repeatability of this assay.
In the future, further research is needed to assess the dose response relationship at low concentrations and to evaluate the metabolic activities (cytockine production) of the lymphocytes influenced by these extracts.

The research was supported by FONDAZIONE CARIVERONA (call 2004 – VALAMAL project) and MIUR (PRIN 2005). We are grateful to INDENA for supplying the vegetal extracts.

REFERENCES – **Gillies,** R.J., Didier, N., Denton, M., 1986. Determination of cell number in monolayer cultures. Anal. Biochem. 159: 109-113. **Hansen**, M.B., Nielsen, S.E., Berg, K., 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods 119: 203-210. **Magaud**, J.P., Sargent, I., Mason, D.Y., 1988. Detection of human white cell proliferative responses by immunoenzymatic measurement of bromodeoxyuridine uptake. J. Immunol. Methods 106: 95-100. **Messele**, T., Roos, M.T.L., Hamann, D., Koot, M., Fontanet A.L., Miedema F., Schellekens, P.T.A., de Rinke Wit T.F., 2000. Nonradioactive techniques for measurement of *in vitro* T-cell proliferation: alternatives to the [3H]thymidine incorporation assay. Clin. Diagn. Lab. Immunol. 7: 687-692. **SPSS** Base 14.0 User's guide. 2005 SPSS inc, pp 421-438. **Tuszynsky**, G., Murphy, A., 1990. Spectrophotometric quantification of anchorage-dependent cell numbers using the bicinchoninic acid protein assay reagent. Anal. Biochem. 184: 189-191.