Effect of doxorubicine on the immunological system in mice

JANINA DROZD, ELŻBIETA L. ANUSZEWSKA

Biochemistry and Biopharmaceuticals Department, National Medicines Institute, Warsaw, Poland

Abstract

Immunomodulatory activity of doxorubicine was assessed with the use of two screening tests: test of cytotoxicity and test of hemagglutination. Substance of proved immunosuppressive properties, that is cyclosporine A was used for comparison. The obtained results show that doxorubicine increases survival rate of mouse thymocytes in cultures with hydrocortisone. Agglutination titre of mouse serum (active hemagglutination test) following doxorubicine injection evidenced a distinct reduction in immunoglobulins representing IgM+IgG and IgG classes in comparison to the control group, which received buffered saline solution. Doxorubicine at the applied concentrations stimulates cell response while inhibiting humoral response of the mouse immunological system.

Key words: *doxorubicine, cyclosporine A, immunomodulation.*

(Centr Eur J Immunol 2010; 35 (2): 53-57)

Introduction

Due to their broad spectrum of activity, antibiotics representing the anthracycline group are of special importance in cancer therapy. They are used for treating both diseases of the haematopoietic system and solid tumours. Cytotoxic effects of anthracycline antibiotics are multidirectional: they block the cleavable DNA-topoisomerase II complex, induce DNA strand breaks, form adducts, intercalate between DNA strands and act through the free radical mechanism [1-3].

There is a growing body of information on immunosuppressive activity of anthracycline activity, which may have adverse impact on the course of anticancer therapy. For example, daunorubicine was shown to inhibit activity of lymphocytes B in mice [4]. However, administration of the combination of doxorubicyne and the GM-CSF agent makes it possible to overcome cancer toleration in transgenic mice and induce specific response against tumour [5], also administration of doxorubicine along with cytokines (IL-10, IL-2, INF-γ) results in inhibition of tumour development [6,7].

There are two types of immunological responses recognised in a living organism: of the cellular and humoral types. As regards cellular response, immunological competent cells (lymphocytes T) directly interact with an antigen, whereas in the case of humoral response, antibodies (immunoglobulins) produced by lymphocytes B take part in the response. Usually, antigens cause simultaneous responses of both types and which of them dominates depends on the form, manner of administration and sensitization path.

The study was aimed at determination of the effect of doxorubicine representing anthracycline antibiotic group on the mouse immunological system and comparing its action with that of the classic immunosuppressive substance, that is cyclosporine A, with the use of the active hemagluttination-humoral response test and cytotoxicity-cellular response test. Two methods for determination of cell survival were applied in the cytotoxicity test: *in vivo* staining with trypan blue and microscopic assessment and cytometric measurement with annexin V.

Materials and methods

Animals

Mice of the Balb-c inbred strain, females, four-week old, weighing approximately 16 g (cytotoxicity test) and 6-8 week old weighing approximately 20 g

Correspondence: Elżbieta Anuszewska, Biochemistry and Biopharmaceuticals Department, National Medicines Institute, Chełmska 30/34, 00-725 Warsaw, Poland, phone number: +48 22 841 54 31, e-mail: anuszewska@il.waw.pl

(hemagglutination test) were used for experiments. The animals were obtained from the Animal Laboratory of the National Institute of Public Health.

Experiments on animals were approved by the Bioethics Committee (approval No. 18/2003) (7/NIZP/2005).

Culture media

Culture medium RPMI-1640 containing Hepes 20 mM/l, IITD Wrocław.

Buffered saline solution, PBS, IITD Wrocław.

Reagents

Hydrocortisone – (Corhydron) (HC) 100 mg, powder and solvent, manufactured by Jelfa.

Sheep red blood cells (SRBC), Biomed, stabilised in Alsever's solution.

Alsever's solution: glucose (POCH), sodium citrate (POCH), sodium chloride (POCH), citric acid (POCH).

Foetal calf serum, Bioprodukt.

Trypan blue, substance, manufactured by Merck.

2-mercapto-ethanol (DMSO), solution, manufactured by Sigma.

Cyclosporine A (CSA), substance, manufactured by Novartis.

Doxorubicine hydrochloride (DOX) (Adriblastine RD, 50 mg), manufactured by Pharmacia&Upjohn.

Annexin-V FITC Apoptosis Kit, BioSource Europe S.A.

Methods

The weighted amount of doxorubicine was dissolved in water, diluted to the appropriate concentration and filtered through microbiological filter paper. Solutions were prepared to obtain final concentrations: 58 and 116 μg/ml of culture. DOX concentrations ranging from 0.58 μg/ml to 174 μg/ml were selected based on the results of cytotoxicity studies.

Weighted amount of cyclosporine A was dissolved in 70% ethanol and filtered through microbiological filters. Solutions were prepared to obtain final concentrations: 58 and 116 μg/ml of culture. CSA concentrations were selected based on the literature data [8, 9].

Solutions of the substances under analysis were added in 10 μl volumes. Adding 10 μl/ml of 70% ethanol to thymocyte culture did not affect their survival (reagent control).

Cytotoxicity test

The test was carried out under sterile conditions. Four-week old mice were killed with chloral hydrate. The thymuses were obtained under sterile conditions and then dissociated on a stainless steel sieve screen (opening diameter size approx. 300 μm, manufactured by Sigma). Thymocytes originated from dissociated thymuses were flushed three times with RPMI-1640 and then centrifuged (1800 rpm for 7-8 min). After the final centrifuging, thymocytes at the concentration of 2×10^6 cells per 1 ml were suspended in RPMI with addition of 10% foetal calf serum. Thymocyte suspension was put into sterile 1 ml tubes which were supplemented with doxorubicine so that concentrations of 58 and 116 μg/ml were obtained and cyclosporine A to attain the concentrations of 58 and 116 μg/ml of culture. After one hour hydrocortisone was added at the concentration of 50 μg/ml of thymocyte culture. Thus prepared samples were incubated for 18-20 h (microscope assessment) or 4-6 h (cytometric assessment) at the temperature of 37°C and in CO_2 atmosphere.

To 18-hour-old thymocyte cultures, 0.04% solution of trypan blue was added and then the number of live cells was determined with the use of Labophot-2A, Nikon microscope. The results are presented as a proportion of live cells in relation to the control culture.

The cultures were prepared for the cytometric measurement in an ice bath. The cultures were centrifuged for 5 min at 1800 rpm. Supernatant was removed and 1 ml of PBS was added to the cell sediment. Content of each tube was thoroughly mixed, centrifuged again and 1 ml of 1% Annexin-V Binding Buffer was added to the sediment and thoroughly vortexed. Polystyrene cytometric tubes were filled with 100 μl of cell suspension, 5 μl of annexin-V solution and 10 μl solution of propidine iodide. Then they were incubated for 15 min in darkness, supplemented with 400 μl of 1% Annexin-V Binding Buffer. Then the cytometric measurement was performed with the use of FACS Vantage Cytometer, Becton Dickinson Co. with CELL Quest 3.3. software.

In a correctly conducted experiment, the absolute number of live thymocytes in a control culture should exceed 75%.

Active hemagglutination test according to Adler [10] as modified by the authors [11]

Six-eight-week-old mice were administered intraventrically a single dose of 4×10^8 of sheep red blood cells (SRBC). A single dose (recognised as an optimal one) of 58,0 μg/mouse of DOX or CSA was administered subcutaneously for 5 successive days. On the 7th day after immunisation, mice were sacrificed with the use of chloral hydrate, blood was collected and serum was separated. Collected blood was put into a heater for 15 min and then kept at the temperature of 4°C for 30 min and then clot was cut off. Samples were centrifuged at 2500 rpm for 10 min and serum was collected from each sample. Serum was placed in a water bath at 56°C for 30 min to inactivate complement. Then serum with no complement was divided into two groups, A and B. Serum representing the A Group was exposed to 0.1 M DMSO for 30 min at 37°C to destruct IgM class immunoglobulin. 1% SRBC suspension was added to successive dilutions of serum representing A and

B groups prepared on microslides and they were incubated for 2 h at 37°C and then stored for 18-20 h at 4°C.

The highest concentration of serum at which agglutination still takes places (microscope assessment – at least three clumps in the field of view at 200 × magnification) was recognised as serum agglutination titre (estimated number of antibodies in serum).

In Group A serum (subjected to DMSO action) the level of IgG class antibodies was determined and in Group B serum the level of IgM and IgG antibodies in total was determined.

All the results obtained were converted into logarithms of inversions of dilutions at which agglutination still occurred and subjected to a statistical analysis with the use of Medistat software [12] to obtain the mean standard error (SE) and statistical significance of differences for two associated samples.

Results

Table 1 presents results of comparison of DOX and CSA effects on survival of mouse thymocytes in cultures with hydrocortisone as determined with the cytotoxicity test:

– with microscopic assessment with trypan blue,

– with the method of flow cytometry.

The results obtained with the cytometric method confirm the microscope assessment with trypan blue showing that presence of DOX results in reduction in cytotoxic effect in the cultures with hydrocortisone, whereas CSA has no effect on survival of mouse thymocytes in the culture with hydrocortisone at the concentration of 58 μg/ml and reduces the number of live cells at the concentration of 116 μg/ml.

Statistically significant differences in values of individual results in some of experimental systems most probably result from precision of the applied methods for assessment of thymocyte survival. Selection of the optimum method for identification of live and dead cells depends on the cell type, character of an agent causing cell death, death type, quality of required information and technical restrictions [13, 14]. Numerous methods for analysing cell capability to survive are based on changes in properties of the cytoplasmatic membrane. Intact membrane of living cells is capable of expelling positively charged dyes such as trypan blue (used for microscope assessment in the cytotoxicity test), propidine iodide (PI) and 7-amin-actinomycin-D. Short incubation of cells with those dyes allows for specific marking of dead cells and those at the late stage of apoptosis as – contrary to live cells – they are not able to expel dyes and are characterised by intense staining [15]. The test of propidine iodide expelling is a cytometric equivalent to the test of trypan blue exclusion used in light microscopy. In comparison to other methods used for quantitative identification of live cells such as morphological analysis or DNA electrophoresis, flow cytometry is a fast, much more sensitive and objective method [16, 17]. The method is commonly used in immunology and oncology for studying issues related to immunotyping of cells, studying their functional state, analysis of haemopoietic cells, assessment of DNA ploidy and apoptosis. Cytometric study makes it possible to discriminate live cells from apoptotic and necrotic ones and determine the actual count of live cells.

Table 2 presents an example of proportions of individual thymocyte fractions in the whole population: control population and after administering HC, DOX or CSA.

Table 3 includes results produced by the active haemagglutination test, which shows a statistically significant decline in immunoglobulin of the IgM + IgG class and IgG class in blood serum of mice which were given DOX at the dose of 58.0 μg/mouse in comparison to the control group which received buffered saline solution. In blood serum of mice which received CSA at the dose of 58 μg/mouse, a statistically significant increase in immunoglobulins of IgM and IgG and IgG classes in comparison to the control group was recorded. Figure 1 graphically presents percentage changes in the level of immunoglobulins in the serum of mice, which received DOX or CSA.

Table 1. Effect of DOX and CSA on survival of mouse thymocytes in cultures with HC. Cytotoxicity test with final microscope assessment after staining with trypan blue and with cytometric assessment with annexin V

Final assessment type	Microscope assessment with trypan blue	Cytometric assessment with annexin V
DOX 58 μg/ml of culture	141.4 ±1.99% *n* = 24*	131.8 ±0.75% *n* = 3
DOX 116 μg/ml of culture	124.6 ±2.90% *n* = 32*	130.0 ±1.58% *n* = 3
CSA 58 μg/ml of culture	96.3 ±3.31% *n* = 5	101.5 ±1.16% *n* = 3
CSA 116 μg/ml of culture	93.1 ±2.57% *n* = 12 **	91.3 ±1.30% *n* = 3

* $p < 0.0001$
** $p < 0.05$
The control (100%) was thymocyte culture with HC in which the survival rate was 59.52%.

Table 2. Percentages of individual thymocyte fractions in the whole population: control population and after administering HC, DOX, CSA (cytogram data)

Cell number	Control	HC 50 µg/ml	DOX 58 µg/ml	CSA 58 µg/ml
live	89.28	59.52	78.58	60.54
at early apoptosis	0.02	24.34	11.32	17.30
at late apoptosis	0.00	15.80	9.70	20.72
nekrotic	10.70	0.34	0.40	1.44

Table 3. Effect of DOX and CSA on the level of immunoglobulins expressed as mean log2 of dilution at which agglutination ± SE was recorded

Immunoglobulin class	Control	DOX 58 µg/ml	CSA 58 µg/ml
IgM + IgG	7.7 ±0.12 *n* = 7	6.6 ±0.74 *n* = 8	8.2 ±1.47 *n* = 9
IgG	7.2 ±0.09 *n* = 6	6.3 ±0.71 *n* = 8	7.4 ±1.40 *n* = 7

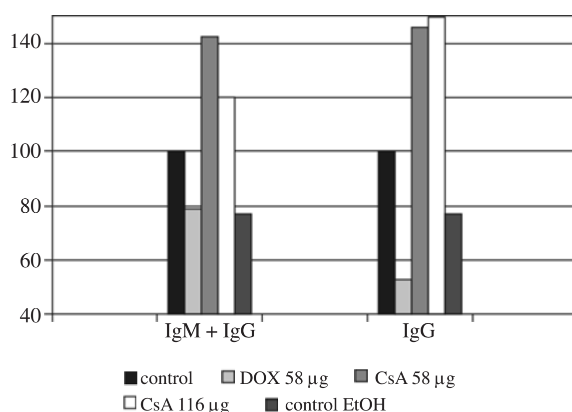

Fig. 1. Proportion of immunoglobulins in mouse blood serum after administering DOX or CSA

Immunomodulating effects may be divided into stimulating and suppressive ones. Such a division means simplification as some agents act on immunological response in both stimulating and suppressive manners, depending among others on the dose level, application scheme, research system (*in vivo*, *in vitro*) or the state of organism immunological reactivity.

The cytotoxicty test performed with the use of mouse thymocyte culture makes it possible to assess the effect of an exogenic agent on the thymus, the central link of organism's cell response. T lymphocytes developing in the thymus under influence of substances stimulating the process of their maturation acquire capabilities related to their role in the matured form. Agents accelerating thymocyte maturation cause among others reduction in density of surface receptors for steroids. As a result they become steroid-resistant and survive in spite of the presence of steroid, which usually induces their apoptosis. Addition of DOX at both concentrations to culture of thymocytes increased statistically significantly their survival, irrespective of the applied method for the final assessment (see Table 1). Thus, it stimulates the immunological cell response. CSA, being substance of confirmed suppressive effects, inhibited the cell response but only slightly and reversely proportionally to the concentration applied.

In the active hemagglutination test, the reaction of specific binding of antibodies with an antigen in the form of ram erythrocytes is used. When cells bind with specific antibodies, they form complexes and drop from reaction suspension in the form of "lints". In the original humoral response, in which a virgin, genetically determined immunological clone of competent cells contacts a thymus-dependent antigen, production of IgM class immunoglobulins is recorded and only then IgG class immunoglobulines appear. A typical property of a mammal organism is that surface immunoglobulins mainly of the M type occur on the surface of the most of matured inactive B lymphocytes. They are also released to body fluids as the first reaction to an antigen (antigens of the ram erythrocyte surface). Along with the development of immunological response, the amount of IgM declines and the amount of more specific IgG immunoglobulin grows.

Table 3 and Figure 1 show that DOX acts suppressively on mouse humoral response which is evidenced by a decline in immunoglobulins of both classes: IgM + IgG and IgG.

That confirms the published data [6, 7] that anthracycline antibiotics may suppress activity of B lymphocytes. In contrary, CSA had a stimulating effect on mouse humoral response and a suppressive effect on cell response.

The results obtained for DOX, stimulation of cell response and inhibition of humoral response confirm the necessity for assessment of effects of the substance under study on immunological activity with the use of several independent tests, both *in vitro* and *in vivo* to obtain a credible response. At the next stage of the study, we plan to extend the range of tests for precise assessment of doxorubicine immonotropic activity.

References

1. Binaschi M, Capranico G, Dal Bo I, Zunino F (1997): Relationship between effects and topoisomerase II-mediated double-stranded DNA breaks produced by anthracyclines with different sequence specificity. Mol Pharmacol 51: 1053-1059.
2. Coullinane C, Cutts SM, Van Rosmalen A, Phillips DR (1994): Formation of adriamycin-DNA adducts in vitro. Nucleic Acid Res 22(12): 2296-2303.
3. Fornari FA, Randolph JK, Yalowich JC et al. (1994): Interference by doxorubicin with DNA unwinding in MCF-7 breast tumor cells. Mol Pharmacol 45: 649-656.
4. Seminara P, Franchi F, Konovalova N et al. (2001): Activity of a nitroxylated analog of daunorubicin, ruboxyl, in B-lymphoproliferative disorders, Acta Haematol 105(2): 77-82.
5. Machiels JP, Reilly RT, Emens LA et al. (2001): Cyclophosphamide, doxorubicin and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines In HER-2/neu tolerized mice. Cancer Res 61: 3689-3697.
6. Kitani A, Fuss I, Nakamura K et al. (2003): Transforming growth factor (TGF)-beta1-producing regulatory T cells induce Smad-mediated interleukin 10 secretion that facilitates coordinated immunoregulatory activity and amelioration of TGF-beta1-mediated fibrosis. J Exp Med 198: 1179-1188.
7. Elbayoumi TA, Torchilin VP (2008): Tumor-specific antibody-mediated targeted delivery of Doxil reduces and manifestation of auricular erythema side effect in mice. Int J Pharm 357 (1-2): 272-279.
8. Podlewski JK, Chwalibogowska-Podlewska A. Leki współczesnej terapii. Split Trading 2003/2004.
9. Pharmindex Kompendium Leków, CMP Medica Poland, Warszawa 2006.
10. Adler FL (1965): Studies on mouse antibodies. I. The response to sheep red cells. J Immunol 95: 26-38.
11. Sawicka T, Prosińska J, Drozd J (1997): Wpływ wybranych kwasów porostowych na odpowiedź komórkową i humoralną układu immunologicznego myszy. Biuletyn Instytutu Leków 41: 17-24
12. Sęk S, Skierski J. Wprowadzenie do metod statystycznych, teoria i praktyka – mikrokomputerowy system Medistat. CMKP, Warszawa 1990.
13. Rotman B, Papermaster BW (1966): Membrane properties of living mammalian cells as studies by enzymatic hydrolysis of fluorogenic esters. Proc Nat Acad Sci U S A 55: 134-141.
14. Darżynkiewicz Z, Bruno S, Del Bino G (1992): Features of apoptotic cells measured by flow cytometry. Cytometry 13: 795-808.
15. Zamai L, Falcieri E, Marhefka G, Vitale M (1996): Supravital exposure to propidium iodide identifies apoptotic cells in the absence of nucleosomal DNA fragmentation, Cytometry 23: 303-311.
16. Darżynkiewicz Z, Li X, Gong J (1994): Assays of cell viability: discrimination of Wells dying by apoptosis. Meth Cell Biol 41:15-38.
17. Majno G, Joris I (1995): Apoptosis, oncosis and necrosis. An overview of cell death. Am J Pathol 146: 3-15.