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CFSE flow cytometric quantification of lymphocytic proliferation in extracorporeal photopheresis: Use for quality control

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ABSTRACT

Quality control is essential to validate extracorporeal photopheresis (ECP) processes. There is just one protocol based on PHA-induced proliferation. Since it involves the use of radioactive thymidine, we developed another technique using CFSE labeling. We compared the two tests in a paired series including 18 procedures. The thymidine test was valid. Once proliferation was obtained (10 patients out of 13), the CFSE test was in close agreement with it. In particular, two cases of residual proliferation after ECP were simultaneously detected by both techniques. Only the CFSE test allows targeted analysis of lymphocytes, thus identifying a surviving lymphocytic sub-population.

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1. Introduction

Extracorporeal photopheresis (ECP) is a technique of cellular therapy that was first developed by Edelson et al. in 1987 [1]. Recognized indications for ECP are clonal pathologies of the T lymphocyte [2,3], in particular cell T cutaneous lymphomas of the Sézary type, graft versus host disease, and transplantation rejections of solid body and autoimmune or dermatological diseases such as lichen planus, atopic dermatitis and scleroderma. There are two techniques for performing ECP [4]. UVAR XTS, the American technique, in which all stages are performed in a single apparatus, and the method of Vilbert–Lourmat, which is used in our center, and which combines the use of a Cobe Spectra cellular separator with an UV-MATIC irradiator. In both techniques, there is an ex vivo stage for mononuclear cell treatment. Qualification of the ECP bag is thus essential to validate the whole of the process. Currently, there is no general agreement on which protocol to adopt. The tests used must be reproducible, reliable, and in the future standardizable from one center to another. Quality control must be multidisciplinary involving bacteriological, hematologic and immunological departments. Only the immunological analysis will be developed in this study. There is only one published report of a protocol developed to validate ECP techniques [5]. The protocol, based on the measurement of the inhibition by ECP of lymphocyte T proliferation induced by phytohemagglutinin A (PHA), involves the use of radioactive products. In order to avoid the drawbacks related to their use such as cost, safety and the need for approval of use, we developed an alternative method of flow cytometry with CFSE (5,6-carboxy fluorescein diacetate succinimidyl ester) labeling [6–8]. CFSE is a

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green fluorochrome, that is retained in the cells for several weeks, without inducing problems of viability (at suitable concentrations). During each cellular division, CSFE is distributed in a strictly dichotomic fashion between both daughter cells. Cytometric analysis of its decrease thus makes it possible to follow cell proliferation. We applied this principle to develop a test of lymphocytic proliferation that could be used for quality control.

Our study was divided into three stages. First, we collected all laboratory results obtained by the tritiated thymidine technique, a total of 142 procedures. Concomitantly, we developed and tested a novel cytometry technique using CFSE. Last, we compared the results of the two tests in 18 procedures.

2. Materials and methods

2.1. Patients

2.1.1. Thymidine test

Thirty-four patients were included in the study. They underwent a total of 142 procedures. The most common indication for ECP treatment was GVHD (15 cases) followed by Sézary's lymphoma (9 cases). The remaining patients had various disorders including renal transplant rejection (2 cases) and dermatological diseases (Table 1).

2.1.2. CFSE test

In 18 procedures (13 patients) chosen randomly, the traditional technique with tritiated thymidine was tested in parallel with a method developed in our laboratory using CFSE (Table 1). The two main indications for treatment were, again, GVHD and Sézary's lymphoma but this time with a preponderance of the latter (3 and 5 cases, respectively).

2.2. Process of extracorporeal photopheresis

Photopheresis was performed according to the technique of Vilbert–Lourmat using a COBE Spectra (Gambro BCT, Lakewood, CO, USA) cell separator [4]. Treatment of 1.5 PV of whole blood ensured the collection of approximately 120 mL of buffy-coat. Mononuclear cells were adjusted to 300 mL in physiological salt solution. Soluble 8-MOP was injected into the bag. After measurement of the hematocrit, the cellular suspension was sterilely transferred to an EVA bag (Maco-Pharma, France), which was then placed in an UVA irradiator at 2 J/cm^2 (UV-MATIC irradiator, Vilbert-Lourmat, France). The hematocrit was measured before dilution in the collection bag (XE 2100, Sysmex, Roche, France) and then after dilution before irradiation in an EVA bag. Cells were then infused as soon as possible. For quality control, one sample was collected before irradiation and another after (designated pre-ECP and post-ECP, respectively). These samples were immediately sent to the laboratory, where they were kept at +4 °C in the dark for analysis until the following day.

Table 1
Patient disease.

Disease	Patient number		Test number	
	Thymidine	CFSE	Thymidine	CFSE
GVHD	15	3	50	3
Sézary's	9	5	48	10
Renal transplantation rejection	2	1	14	1
Lichen planus	2	1	18	1
Atopic dermatitis	2	1	3	1
Systemic sclerosis	1	0	3	0
Hemosiderosis	1	1	3	1
Acute leukemia	1	0	2	0
Nephrogenic fibrosing dermopathy	1	1	1	1
Total	34	13	142	18

2.3. Test of lymphoblastic transformation with incorporation of tritiated thymidine

Quality control was based on the measurement of the antiproliferative effect of ECP [5]. Lymphocytes are unable to proliferate spontaneously in culture and hence we performed a preliminary procedure in which proliferation was induced in vitro by PHA (phytohemagglutinin A), a plant lectine capable of activating T lymphocytes without antigenic specificity. After red blood cell lysis with NH4Cl and slow centrifugation, the cells were adjusted to a concentration of 1×10^6/mL in complete RPMI 1640 (Cambrex, BE 12-167F), i.e. with 1% L-glutamine (Sigma, G7513) and 10% fetal calf serum or FCS (Cambrex), without antibiotic. One hundred microliters of the cell suspension was deposited per well in a 96 well sterile plate. Six wells per sample (pre- and post-ECP) were used, including three "control" wells ("control" triplicate designated PHA−) and three "PHA" wells ("PHA" triplicate designated PHA+). Five microliters of PHA 0.2 mg/mL (Sigma, L9312) were added to each PHA+ well. Cells were kept 72 h in culture at 37 °C under 5% CO_2. 1 μCi of tritiated thymidine (Amersham Biosciences, TRK 418, France) was added to each well during the 4 last hours of cell culture. Cells were then transferred onto Whatman paper using a cell harvester. Tritiated thymidine incorporation was then measured using a β counter. Proliferation results were expressed in cpm (counts per minute) as the mean value of the triplicates. The percent inhibition was calculated as follows [5]: 100 − (100 × cpm after/cpm before ECP).

2.4. Nonradioactive alternative: test of PHA-stimulated lymphocytic proliferation with CFSE labeling

We compared PHA-induced proliferation on UVA-irradiated cells and on non-irradiated cells after three days of culture. Our aim was to measure the degree of inhibition of proliferation induced by ECP. The mitotic activity of the T lymphocytes was assessed from the number of cell divisions. We used the same samples in parallel for both techniques. The first part of the procedure was the same as that described for the tritiated thymidine technique. The differences between the two procedures occurred only after counting (with trypan blue). In our alternative technique, mononuclear cells were adjusted to a concentration of 10×10^6/mL. The PHA stimulation methods were the same

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