

Lymphocyte proliferation response in patients with delayed hypersensitivity reactions to heparins

S. Lopez, M.J. Torres,* R. Rodríguez-Pena, N. Blanca-Lopez,† T.D. Fernandez, C. Antunez, G. Canto,‡ V. de Luque‡ and C. Mayorga

Research Laboratory, Carlos Haya Hospital-Fundación IMABIS, 29009 Malaga, Spain

*Allergy Service, Carlos Haya Hospital, Malaga, Spain

†Allergy Service, 12 de Octubre Hospital, Madrid, Spain

‡Allergy Service, Virgen Macarena Hospital, Seville, Spain

Summary

Correspondence

Cristobalina Mayorga.

E-mail: mayorga.lina@gmail.com

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Conflicts of interest

None declared.

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Background Heparins can induce delayed-type hypersensitivity (DTH) reactions mediated by specific T lymphocytes. However, the interaction between heparins and lymphocytes has not been sufficiently studied.

Objectives To analyse the lymphocyte response to heparins using different types of antigen-presenting cells in patients with DTH reactions to these drugs.

Methods We studied seven patients with DTH reactions to heparins diagnosed by delayed reading of intradermal skin testing ($n = 5$) or drug provocation test ($n = 2$) and nine tolerant controls. Biopsies were obtained from intradermal testing or during the acute reaction. Peripheral blood mononuclear cells were used to obtain T and B lymphocytes, monocytes and monocyte-derived dendritic cells (DC). T-lymphocyte proliferation was assayed by means of ^3H -thymidine incorporation.

Results Skin testing showed a high degree of cross-reactivity within low molecular weight heparins with good tolerance to sodium heparin, fondaparinux and lepirudin in most cases. The proliferative response was positive in six patients to most of the heparins tested with both monocytes and B cells (the classical lymphocyte transformation test) or immature DC as antigen-presenting cells, giving a higher response with DC. At a second evaluation 1 year later, the proliferative response was found only with DC, and mainly to the culprit drug.

Conclusions A model using DC in the lymphocyte proliferation test is a more appropriate way to assess the immunological response in DTH to heparins; additionally, it can detect a response over a longer time. These findings may be useful for the diagnostic evaluation of drug reactions.

Heparins are anticoagulant drugs widely used in the prophylaxis and treatment of thromboembolic disorders.¹ The common structure of heparins is characterized by sulphate proteoglycan chains of different molecular weights. The compounds available for therapeutic use are: unfractionated heparins (UH); low molecular weight heparins (LMWH), generated by fragmentation of polysaccharide chains from UH; heparinoids, which include heparin derivatives, such as highly sulphated polysaccharides, both natural and synthetic; and fondaparinux, which is a synthetic selective inhibitor of factor Xa with a chemical structure identical to heparin.^{1,2} Important cross-reactivity exists between LMWH,^{3–5} with low cross-reactivity with the synthetic fondaparinux, although higher than previously thought.⁶ No cross-reactivity with

hirudins, a group of new anticoagulants, has been detected, although they have been associated with a high rate of anaphylaxis, sometimes lethal.⁷

The chemical structure of heparins, composed of polyvalent anions, gives the drugs a high capacity to bind to proteins and, theoretically, to form conjugates that can induce specific immune responses.¹ Despite this, and bearing in mind that heparins are commonly used drugs, hypersensitivity reactions to this structure are uncommon.³ One reason may be their anti-inflammatory properties, which have been reported to have clinical benefits in the treatment of asthma⁸ or to prolong the survival of allografts in some experimental models.⁹ Proposed mechanisms include the inhibition of interferon- γ responses,^{9,10} downexpression of P- and L-selectin,¹¹ the

binding of IP-10, I-TAC and Mig, or the disruption of CXCR3 Th1 cell trafficking.¹² Another reason could be the possibility that exogenous heparins may avoid the immune responses by means of tolerance mechanisms developed against the endogenous homologous proteins.

Nevertheless, some patients treated with heparins have experienced allergic reactions,³ which can be delayed-type hypersensitivity (DTH) cell-mediated reactions (which form the subject of this study) or immediate antibody-mediated reactions. Manifestations like pruritus, urticaria, rhinoconjunctivitis, bronchial asthma and anaphylaxis have been attributed to preservatives in the preparations, although with the progress in pharmaceutical processing they are now extremely rare.¹³ Clinical manifestations of DTH reactions to heparins include erythematous plaques at the injection site, maculopapular exanthema (MPE) and more severe symptoms, like skin necrosis.¹⁴ Regarding the mechanisms involved, no description of specific IgE responses exists and studies based on the lymphocyte transformation test (LTT) are usually negative.¹⁵ This has been attributed to the anti-inflammatory effects of heparins.^{3,16}

The aim of this work was to set up an *in vitro* system to evaluate subjects with DTH to heparins by using dendritic cells (DC), the most potent antigen-presenting cells (APC) in the immune system. We obtained specific *in vitro* proliferation against heparins, with or without DC, in patients who had experienced a delayed allergic reaction, but not in controls who used and tolerated the drug. The use of DC improved the lymphocyte stimulation induced by heparin, suggesting that these cells are able to present the hapten more efficiently.

Materials and methods

Patients and controls

The study was carried out in patients who had had a DTH reaction to heparins over a 3-year period. The diagnosis was established by the clinical history, intradermal skin testing with delayed reading, and a drug provocation test (DPT) if necessary. A skin biopsy was also obtained from the affected skin during the reaction or the positive skin test to confirm an immunological mechanism.

A control group was composed of nine healthy women (mean age 52 years), five of whom had tolerated enoxaparin, three nadroparin and one sodium heparin and nadroparin.

The institutional review board approved the study, and informed consent for all the diagnostic procedures was obtained from all patients and controls.

Reagents and drugs

Anticoagulants tested in skin testing and *in vitro* assays were: (i) UH: sodium heparin (Rovi, Madrid, Spain); (ii) LMWH: dalteparin (Fragmin[®]; Pfizer, Madrid, Spain), bemiparin (Hibor[®]; Rovi), nadroparin (Fraxiparina[®]; GSK, Madrid, Spain), tinzaparin (Innohep[®]; Farmacusi, Madrid, Spain),

enoxaparin (Clexane[®]; Sanofi-Aventis, Barcelona, Spain); (iii) heparinoids: sulodexide (Aterina[®]; Tedec-Meiji Farma, Barcelona, Spain); (iv) fondaparinux (Arixtra[®]; GSK); and (v) lepirudin (Refludin[®]; Schering, Madrid, Spain).

Immunohistochemical analysis was performed with the monoclonal antibodies anti-CD1a, CD4, CD69, CD25, CD57, perforin and granzyme B (Novocastra Laboratories, Newcastle upon Tyne, U.K.), CD3, CD8 and HLA-DR (Dako, Ely, U.K.), CLA (BD Pharmingen, San Diego, CA, U.S.A.), antimouse IgG conjugated to peroxidase-labelled dextran polymer (Zymed Lab., San Francisco, CA, U.S.A.) and 3,3'-diaminobenzidine substrate kit (Sigma, St Louis, MO, U.S.A.).

Allergological tests

The first step was intradermal skin testing, with all the heparins described above at a maximum dilution of 1/10, as described.³ Readings were made at 24, 48 and 72 h. A reaction was considered positive if an infiltrated erythema with a diameter > 5 mm appeared.

If skin-test negative, a DPT was done to confirm the diagnosis, by administering different anticoagulants undiluted and subcutaneously in the abdominal wall, except for sodium heparin which was administered intravenously. We tested just one per week, beginning with 0.2 mL of the undiluted dose and increasing at 1-h intervals, until the therapeutic dose was reached. Patients were followed up to day 5–7, as positive reactions may occur only after several days.

Skin immunohistochemical studies

Four-millimetre punch skin biopsies were obtained during the acute phase of the reactions or from skin-test-positive reactions, fixed in 10% formalin and paraffin embedded. Microtome sections (8 µm) were processed for haematoxylin and eosin and immunohistochemical staining, as described.¹⁷

Cell isolation from peripheral blood and generation of immature dendritic cells

From patients and controls, 40 mL of fresh peripheral blood was obtained in anticoagulated ethylenediamine tetraacetic acid (EDTA) tubes, and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density gradient. A portion of PBMC was used to isolate monocytes by magnetic separation using Miltenyi Biotec (Bergisch Gladbach, Germany) CD14 microbeads. CD14⁺ monocytes were cultured for 5 days at 5% CO₂ and 37 °C in complete medium supplemented with 200 ng mL⁻¹ of recombinant human (rh) granulocyte/macrophage colony-stimulating factor and 100 ng mL⁻¹ rh interleukin-4 to generate immature DC (imDC), as described.¹⁸ A portion of PBMC and the negative CD14 fraction were used to obtain T lymphocytes by magnetic separation using Miltenyi Biotec CD3 microbeads. In the case of PBMC the negative fraction containing monocytes and B cells was used as APC. Both fractions were then frozen in

10% dimethylsulphoxide for later experiments while the DC were being generated.

³H-thymidine incorporation assay for lymphocyte proliferation test

The studies were made in parallel by using monocytes and B cells (the classical LTT) or imDC as APC to stimulate autologous T lymphocytes in the presence of different heparins. This was carried out as described, with a few modifications.¹⁸ T lymphocytes were seeded in triplicate at 1.5×10^5 cells per well on to 96-well, U-bottom plates. The T lymphocyte/APC ratio was 1 : 4 for monocytes and B cells and 1 : 15 for imDC. The negative control was T lymphocytes + APC without drugs and the positive control T lymphocytes + APC with $5 \mu\text{g mL}^{-1}$ tetanus toxoid. Due to differences in heparin preparations, the heparin doses used were adapted in all cases to the same international units (IU). The doses used were 5 and 0.5 IU, selected based on their null cytotoxicity in healthy controls and null inhibitory effect on phytohaemagglutinin-stimulated lymphocytes. After 6 days, $1 \mu\text{Ci}$ of ³H-thymidine was added to each well, and the cells were incubated for an additional 18 h. The cells were then harvested on to glass fibre filters (Skatron AS, Tranby, Norway), and the beta cell-associated radioactivity was assessed by scintillation spectroscopy with a TopCount scintillation counter (Packard, Downers Grove, IL, U.S.A.). Results were detected in counts per minute (c.p.m.) and expressed as the stimulation index (SI), calculated as the ratio of the mean of triplicate c.p.m. of the target sample minus the mean of triplicate c.p.m. of the negative controls to the mean of triplicate c.p.m. of the negative controls. A SI > 3 with any of the doses used was considered positive.

Results

The study included seven women with DTH reactions to heparin; their mean age was 56.6 years and the mean time between the reaction and sample collection was 5.7 months (Table 1). The diagnosis was confirmed in five patients by a

positive intradermal test to the culprit heparin, and in two cases the drug needed to be re-administered to confirm the diagnosis. In all patients with a positive skin test, results were positive at 24–48 h, except patient 7, in whom results were positive at 72 h.

In all patients who were skin-test positive, cross-reactivity between different LMWH was detected and in two this was extended to heparinoids (sulodexide). All patients were skin-test negative and had good tolerance in DPT to sodium heparin, to synthetic fondaparinux and to another antithrombotic drug of a different origin, lepirudin, except patient 6 in whom no DPT was performed. Figure 1 shows a typical positive intradermal test and delayed local reaction.

We obtained skin biopsies from all patients who were skin-test positive and from two patients during the acute phase of the reaction. In all of these, we detected a T-cell infiltrate composed mainly of CD4 and, to a lesser extent, CD8 lymphocytes. The T cells were activated, expressing CD25 and HLA-DR, with no detection of the early activation marker, CD69. Cells expressed high levels of CLA and were localized to the upper dermis and lower level of the epidermis. Cytotoxic markers (perforin and granzyme B) were either not detected or were detected to just a low degree. Other findings were the presence of Langerhans cells in the epidermis and natural killer cells in the dermis (Fig. 2).

We analysed the specific proliferative response by using monocytes and B cells (the classical LTT) or imDC as APC. In six patients, including the two who were skin-test negative (patients 2 and 4), there was a positive lymphocyte proliferation against different heparins tested with the two APC, although the SI was higher when we used DC (Fig. 3). In all patients and controls the LTT with sodium heparin, fondaparinux and lepirudin was negative (data not shown). Neither the classical nor the DC-enriched cultures showed any lymphocyte proliferation in controls.

In two patients (patients 1 and 3) the study was performed twice: during the first month after the reaction (T1) and 1 year later (T2) (Fig. 4). In patient 1, the first *in vitro* analysis showed lymphocyte proliferation against enoxaparin, nadroparin and sulodexide, whichever APC were used. Never-

Table 1 Clinical characteristics, time interval between the reaction and sample collection and the results of the allergological tests

Patient	Age (years)/sex	Reaction	Drug	Interval (months) ^a	Skin-test positive	Skin-test negative	DPT
1	50/F	MPE	EP	1	DP, BP, NP, TZ, EP, SD	SH, FP, LP	SH–, FP–, LP–
2	46/F	MPE	NP	12		SH, DP, BP, NP, TZ, EP, SD, FP, LP	NP+, SH–, FP–, LP–
3	63/F	Local	EP	0.5	BP, NP, EP	SH, DP, TZ, SD, FP, LP	SH–, FP–, LP–
4	60/F	Local	EP	7		SH, DP, BP, NP, TZ, EP, SD, FP, LP	EP+, SH–, FP–, LP–
5	33/F	Local	NP	12	DP, BP, NP, TZ, SD, EP	SH, FP, LP	SH–, FP–, LP–
6	62/F	Local	EP	3	EP, NP	SH, DP, BP, TZ, SD, FP, LP	Not done
7	82/F	Local	EP	2	BP, EP	SH, DP, NP, TZ, SD, FP, LP	SH–, FP–, LP–
		Local	BP	3			

^aTime interval between the reaction and the study. DPT, drug provocation test; MPE, maculopapular exanthema; SH, sodium heparin; DP, dalteparin; BP, bemiparin; NP, nadroparin; TZ, tinzaparin; EP, enoxaparin; SD, sulodexide; FP, fondaparinux; LP, lepirudin.

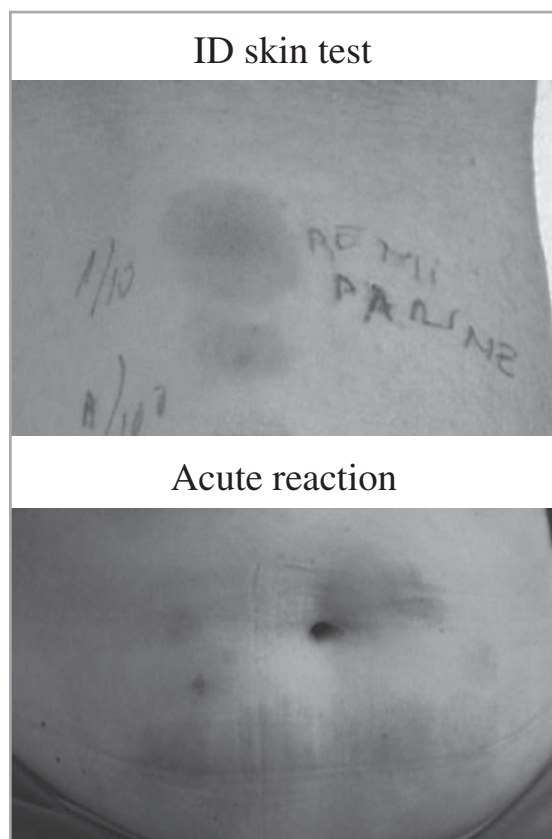


Fig 1. Positive intradermal (ID) skin test to bemiparin at two different concentrations in patient 3 (upper). Local reaction with enoxaparin on abdomen of patient 3 (lower).

theless, in cultures with DC, the SI was higher than in those with monocytes and B cells for the three heparins. In the second evaluation, positive proliferation was detected only to enoxaparin, the culprit drug, and only when DC were used

as APC. Patient 3 showed lymphocyte proliferation with the two types of APC present in the cultures, but slightly higher with imDC in the first evaluation. In the second evaluation, we obtained no proliferation in the classical LTT to any heparin tested, but with DC we observed positive results to nadroparin and enoxaparin, higher with the latter, which was the culprit drug.

Discussion

DTH reactions consisting of erythematous plaques and sometimes MPE are the most common manifestations induced by heparins.³ The skin biopsies obtained from the acute reaction or the positive intradermal test suggested a cell-mediated reaction. The cellular pattern detected was similar to those previously reported in DTH to other drugs,¹⁷ indicating that regardless of the anti-inflammatory effects of heparins, a specific inflammatory T-cell response occurs in the skin after contact with the heparin. Moreover, these findings, especially the lymphocyte infiltrate, enable us to distinguish these reactions from heparin-induced thrombocytopenia.¹⁹

The key point of this study was to evaluate the lymphocyte response to heparins in patients with DTH. This response has been reported to be low and this has been attributed to the self-immunosuppressive characteristic of heparins.^{20,21} However, in our study we observed a specific proliferative response by using both monocytes and B cells (the classical LTT) and imDC as APC in six of seven patients evaluated. This indicates that lymphocytes from patients with DTH responded specifically to heparin, but more efficiently when DC were used, as we have previously found with amoxicillin.¹⁸ These differences in the results may have several explanations. Firstly, the use of heparin-treated tubes to collect blood samples,²¹ which must be rejected in order to avoid the stimulation of T cells from heparin-sensitized patients or to inhibit the proliferative response by the heparin

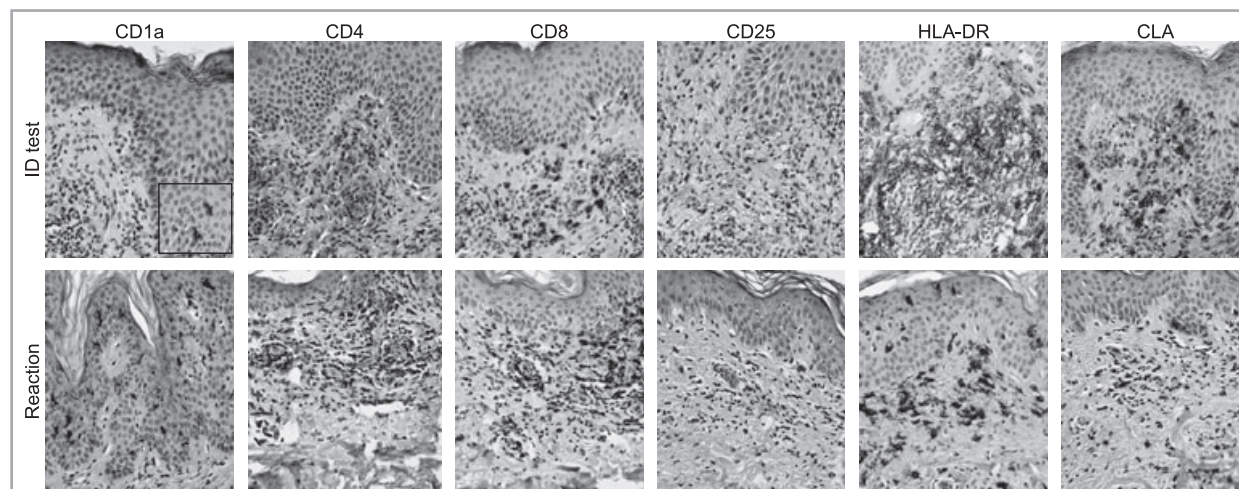


Fig 2. Immunohistochemical analysis of a skin biopsy obtained during the acute phase of the reaction and during the positive intradermal (ID) skin test in patient 3.

Fig 3. Lymphocyte transformation test (LTT) to different heparins (Dp, dalteparin; Bp, bemiparin; Np, nadroparin; Tz, tinzaparin; Ep, enoxaparin; Sd, sulodexide) in seven patients and the control group. The heparin doses used were 5 IU and 0.5 IU. A stimulation index (SI) > 3 with any of the doses used was considered positive. LTTs to sodium heparin, fondaparinux and lepirudin were negative in all cases and controls (data not shown). Mo/Bcell, monocytes and B cells; imDC, immature dendritic cells.

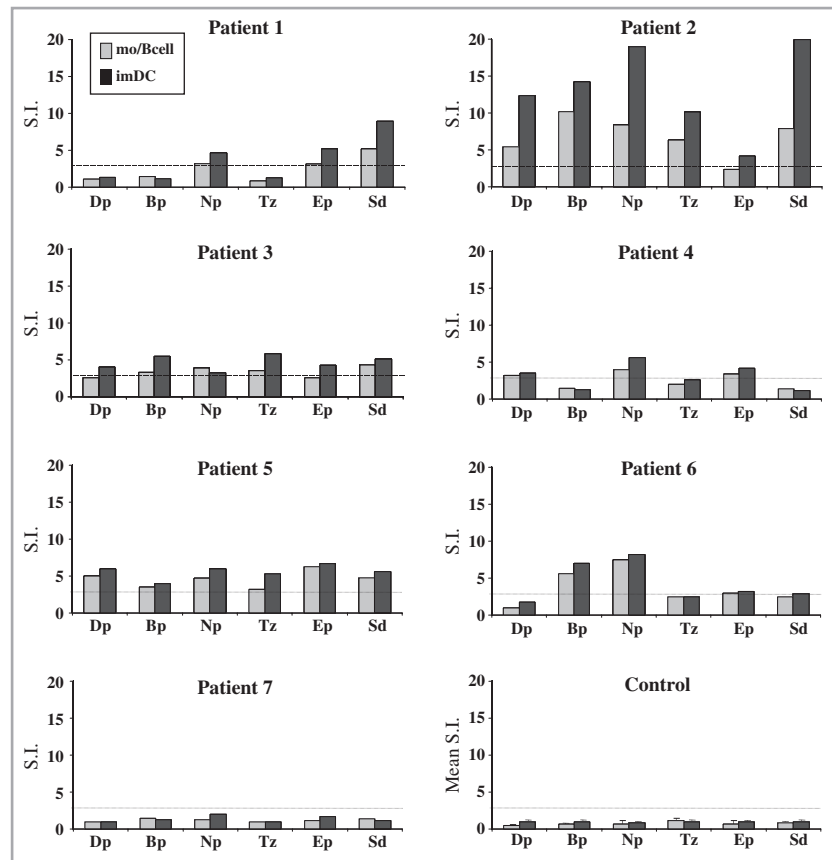
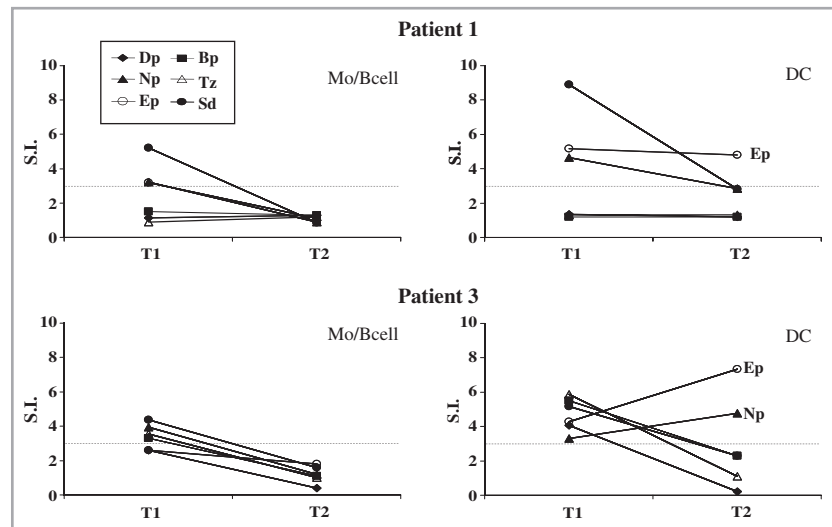


Fig 4. Lymphocyte transformation test to different heparins (Dp, dalteparin; Bp, bemiparin; Np, nadroparin; Tz, tinzaparin; Ep, enoxaparin; Sd, sulodexide) in patients 1 and 3 at two different time points (T1, during the first month after the reaction and T2, 1 year later). Mo/Bcell, monocytes and B cells; DC, dendritic cells.



immunosuppressive effect. For this reason, in our study we used EDTA as an anticoagulant, which proved to be a good substitute in those samples manipulated within 16–18 h after collection. Secondly, the time interval between the occurrence of the reaction and the sample collection could be an important factor and the reason why in our study, with a short time interval, only one patient was negative with the classical LTT. The effect of this factor on the induction of the positive response can be better observed in two cases (patients 1 and

3) where the study was performed very soon after the reaction, during the first month, and also 1 year later. In both patients the first *in vitro* analysis showed lymphocyte proliferation against the culprit LMWH, with a high degree of cross-reactivity, whichever APC was used. Nevertheless, in cultures with DC, the SI was higher than in those with monocytes and B cells for the different heparins. In the second evaluation, positive proliferation was detected only to the culprit drug, and only when DC were used as APC. We thus show

that DC are useful for improving lymphocyte responses, especially after a longer time interval.

The only patient with no proliferation (patient 7) was an old woman (82 years) who had had two episodes, the first with enoxaparin and the second with bemiparin, studied by skin testing and LTT each time, 2 and 3 months after the reaction. On both occasions skin testing was positive at a much delayed reading (72 h) to both bemiparin and enoxaparin. The LTT, with and without DC as APC, was negative for all heparins tested. This case was special, as the patient had a very late skin-test positive response, with lower cross-reactivity and with a negative lymphocyte response. This may be due to the age of the patient, which may have interfered with the specific immune response,²² as it was also detected when tetanus toxoid was used as a positive control (data not shown).

A high degree of cross-reactivity was found between different LMWH, and even in some cases with heparinoids (suloxide), by both skin testing and LTT. Moreover, others have shown that this cross-reactivity does not depend on the molecular weight of these compounds.²³ However, there was no correlation between the lymphocyte response and skin testing, as has been previously shown with beta-lactams.²⁴ As reported by others,²⁵ although the chemical structure of sodium heparin is very similar to LMWH, all our patients were skin-test and LTT negative, and tolerated this drug. This may be due to the fact that sodium heparin is administered intravenously and, as has been reported, intravenous injection of heparin is tolerated despite a DTH response appearing when applied topically. Although there is no clear explanation for this, differences in the absorption of heparin from skin and different processing/presentation of antigens depending on the route could be involved.²⁵ Nowadays, fondaparinux is known to have a higher cross-reactivity with heparins than previously thought,^{3,6} although all our patients were negative in the different tests. In this study we also included lepirudin, an anticoagulant of different origin and, as expected, all patients tolerated the drug. Unfortunately, this drug may not be considered an optimal alternative as it can induce severe anaphylactic reactions on re-exposure.⁷

In summary, we found that the lymphocyte proliferation test using DC as APC can be useful for the evaluation of the immunological response in DTH reactions to heparins. It has the advantage of being able to detect a positive response over a longer period of time than the classical LTT, by preserving the sensitivity to the culprit drug. This finding may be relevant for the *in vitro* evaluation of DTH reactions to heparins.

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