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Platelet agonists and calcium homeostasis in endothelial cells: possible role in the interaction of endothelium with hemostatic system

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ABSTRACT

Background and Objective. **The endothelium is a complex tissue that modulates a vast array of biological functions and Ca^{++} transients are critically important to these endothelium-dependant functions. We addressed the hypothesis that some platelet agonists and products of activation of the hemostatic system could determine Ca^{++} transients in a bovine pulmonary artery endothelial cell line (CPA-47).**

Design and Methods. **The effect of thrombin, collagen, ADP, PAF, PDGF, GRGDS, and the TxA_2 mimetic U46619 on CPA-47-cytoplasmic Ca^{++} transients was evaluated using a Platelet Ionized Calcium Aggregometer, after cells were loaded with the photoprotein aequorin.**

Results. **ADP, GRGDS, PAF, U46619 and collagen were able to induce rapid Ca^{++} transients in CPA-47 endothelial cells and the response was stable after repeated additions, while thrombin acted slightly differently, as cells became refractory to this agonist after the first response, but they remained sensitive to the other inducers. Only PDGF was completely ineffective. Furthermore, calcium-channel blockers verapamil and flunarizine (but not nifedipine) caused a reduction only of thrombin-induced cytoplasmic Ca^{++} transients, while the addition of depolarizing concentration of KCl suggests the presence also of voltage-operated channels on endothelial cell membrane. Finally, EGTA caused the complete suppression of Ca^{++} transients induced by all the tested agonists but collagen.**

Interpretation and Conclusions. **Our study demonstrated that the different agents tested are able to induce Ca^{++} transients on bovine endothelial cultured cells, similarly to that observed in platelets and other non excitable cells, including tumor cells, and that calcium channel blockers had only a limited inhibitory effect on these changes; these results may help more thorough understanding of the biochemical basis of the interaction between endothelium and the hemostatic system.**

Key words: endothelium, Ca^{++} transients, platelet agonists, cell culture, calcium-channel blockers

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The endothelium, which lines the entire circulatory system, is a tissue with a variety of biological functions, and not merely a mechanical barrier with only little metabolic activity, as it was considered in the past.[1]

The endothelium modulates vascular smooth-muscle tone and mediates hemostasis, cellular proliferation, inflammatory and immune mechanisms in the vessel wall; these functions are performed by the endothelium primarily by sensing changes in its environment and responding to the stimuli producing a host of biologically active substances, globally known as endothelium-derived factors.[2] The events associated with blood coagulation are probably the most important triggers for the release of a number of substances from the endothelium; indeed, activated thrombocytes release substances with strong vasoconstrictor properties, e.g., adenosine diphosphate (ADP), 5-hydroxytryptamine (serotonin), thromboxane A_2 (TxA_2), and platelet-activating factor (PAF). These substances can activate specific receptors on the endothelial cell surface, leading to a counteracting release of vasodilatory nitric oxide.[1]

Besides ADP, TxA_2 and PAF, several platelet agonists and products of the activation of the hemostatic system are known to be able to induce and modulate a number of the above endothelial functions; thrombin, for example, evokes several endothelial cell responses which regulate hemostasis, thrombosis and vessel-wall pathophysiology.[3]

The modulation of cytoplasmic Ca^{++} concentration plays a key role in this context, as calcium homeostasis is implicated in the regulation of several important cell functions, such as differentiation,[4,5] receptor expression,[6] release of bioactive substances,[7,8] activation[9-13] and proliferation.[14-16]

In the present study we addressed the hypothesis that the platelet agonists and products of activation of the hemostatic system thrombin, collagen, ADP, PAF, PDGF, the pentapeptide GRGDS, and the TxA_2 mimetic U46619 could determine Ca^{++} transients in normal endothelial cells cultured *in vitro*; furthermore, we studied the repeatability and exhaustibility of Ca^{++} transients after repeated stimuli, together with the effect on these transients of the calcium-channel blockers verapamil, flunarizine and nifedipine.

Materials and Methods

Cell line and culture conditions

The bovine pulmonary artery endothelial cell line CPA-47[17] was purchased from the *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia* (Brescia, Italy). Cells were cultured in Eagle's Minimum Essential Medium in Earle's BSS supplemented with 10% fetal calf serum (FCS), 1% L-glutamine and 1% streptomycin-penicillin. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and passaged once weekly. Confluent monolayers were washed three times with Ca^{++}-, Mg^{++}-free Hanks' balanced salt solution (HBSS) and the cells were harvested in the absence of proteolytic enzymes by exposing the monolayers to 5 mM EGTA in HBSS for 1 hour at 37°C. The cells were then washed three times and resuspended in Ca^{++}-, Mg++-free HBSS, counted in a hemocytometer, and used immediately for the different experiments. Cell viability, as determined by Trypan blue exclusion, ranged from 90 to 96% and remained constant throughout the experiments. Cells were also tested for the absence of *Mycoplasma* with the bis-benzimide staining test.[18]

Endothelial cell cytoplasmic ionized Ca^{++} concentrations

Endothelial cells were loaded with the photoprotein aequorin in a procedure very similar to that usually employed for aequorin loading into platelets,[19] and already used to study Ca^{++} transients in tumor cells.[20] Briefly, endothelial cells were resuspended in a salin-Hepes buffer in the presence of glucose (5.6 mM), EGTA (5 mM) and albumin (0.1%), incubated with aequorin with increasing amounts of dimethylsulfoxide (DMSO), washed twice and resuspended in a Tyrode's buffer with albumin (0.35%), 1 mM Ca^{++} and 1 mM Mg^{++}. In the final suspension endothelial cell concentration was adjusted to 3×10^6/mL. Endothelial cell-cytoplasmic Ca^{++} transients were determined using a Platelet Ionized Calcium Aggregometer (Chrono-Log, Havertown, PA, USA). Aequorin loaded endothelial cells (1 mL) were put into a cuvette in the aggregometer at 37°C. After 3 minutes the agonist was added and changes in luminescence were recorded. The agonists were used at the following final concentrations: ADP 100 µM, GRGDS 0.2 mM, PAF 2 µg/mL, U46619 20 µM, collagen 20 µg/mL, thrombin 10 U/mL, PDGF 0.1 µg/mL.The effect of EGTA, added at the concentration of 2.5 mM, on agonist-induced increase of cytoplasmic Ca^{++} levels, was also investigated.[21]

In other experiments, endothelial cells were preincubated for 3 minutes at 37°C in the aggregometer cuvette with different calcium-channel blockers (verapamil, flunarizine and nifedipine, all at the final concentration of 0.3 mM) before addition of the agonist. Cytoplasmic Ca^{++} levels were calculated assuming an intracellular Mg^{++} concentration of 1 mM.[22]

Chemicals and reagents

Eagle's Minimum Essential Medium, Earles' BSS, FCS, HBSS, and streptomycin-penicillin were purchased from Irvine Scientific (Santa Ana, CA, USA); the L-glutamine, Trypan blue, EGTA, Tyrode's and Hepes buffers, ADP, thrombin, U46619, GRGDS, PAF and PDGF came from Sigma Chemicals (St. Louis, MO, USA), aequorin and collagen was purchased from Mascia Brunelli (Milan, Italy), all plastic material from Falcon (Germany), and the commercial kit for the detection of *Mycoplasma* contamination from Hoechst (Germany).

Results

Agonists-induced Ca^{++} transients in endothelial cells

ADP and the RGD-containing peptide GRGDS were able to induce rapid Ca^{++} transients in CPA-47 endothelial cells and the response was stable after repeated additions (Figure 1).

Ca^{++} fluxes with similar characteristics were induced by PAF, TxA_2-mimetic U46619 and collagen (Figure 2). In contrast, thrombin addition was followed, after a short lag-phase, by a much higher and sustained elevation in Ca^{++} cytoplasmic concentration; endothelial cells became refractory to this agonist after the first response, but they remained sensitive to the other inducers (Figure 3). Of the different substances studied for their ability to increase endothelial cell cytoplasmic Ca^{++} concentrations, only PDGF was never able to induce any change in such levels (data not shown).

These results suggest that a wide spectrum of agents can trigger Ca^{++} transients in endothelial cells by opening distinct receptor-operated channels.

Effects of KCl and EGTA on Ca^{++} transients

Furthermore, the addition of a depolarizing concentration of KCl (50 mM) was followed by a short increase in cytoplasmic Ca^{++} levels, which progressively decreased after repeated additions (Figure 4), which also indicated the presence of voltage-operat-

Table 1. Effect of EGTA (2.5 mM) on agonist-induced increase of cytoplasmic Ca^{++} levels (µM) in endothelial cells (means ± S.E. of 5 independent experiments).

Agonist	*Control*	*EGTA*	*p*
ADP (100 µM)	3.67±1.48	0.00±0	< 0.05
GRGDS (0.2 mM)	3.91±1.65	0.00±0	< 0.02
PAF (2 µg/mL)	2.51±.94	0.00±0	< 0.05
U46619 (20 µM)	2.20±.59	0.00±0	< 0.05
Collagen (20 mg/mL)	4.15±1.25	3.62±1.58	ns
Thrombin (10 U/mL)	6.78±2.07	0.00±0	< 0.02
KCl (50 mM)	2.62±.61	0.00±0	< 0.05

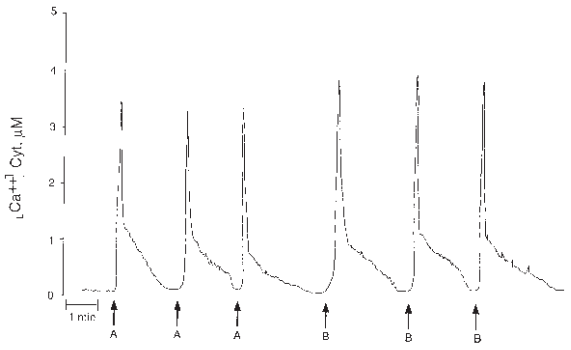

Figure 1. ADP and the RGD-containing peptide GRGDS induce Ca^{++} transients in endothelial cells; the response is stable after repeated additions. A = ADP (100 mM); B = GRGDS (0.2 mM).

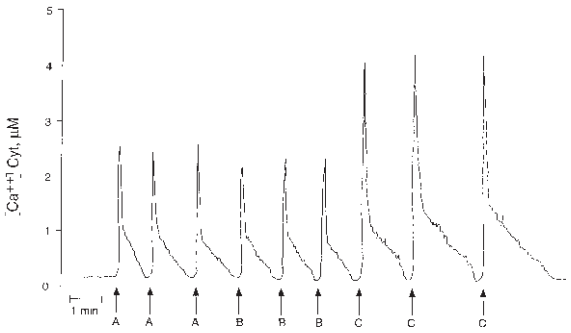

Figure 2. PAF, TxA_2-mimetic U46619 and collagen induce Ca^{++} transients in endothelial cells; again, the response is stable after repeated additions. A = PAF (2 mg/mL); B = U46619 (20 mM); C = collagen (20 mg/mL).

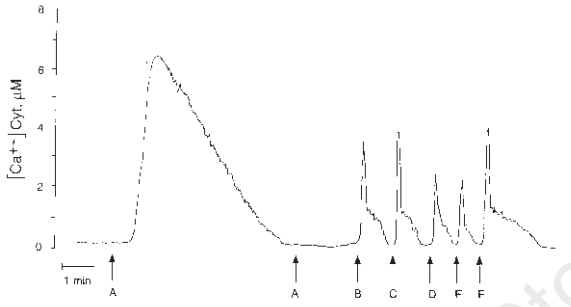

Figure 3. Thrombin addition to endothelial cells is followed by a sustained elevation in Ca^{++} cytoplasmic concentration; endothelial cells are refractory to this agonist after the first response, but they remain sensitive to the other inducers. A = thrombin (10 U/mL); B = ADP (100 mM); C = GRGDS (0.2 mM); D = PAF (2 mg/mL); E = U46619 (20 mM); F = collagen (20 mg/mL).

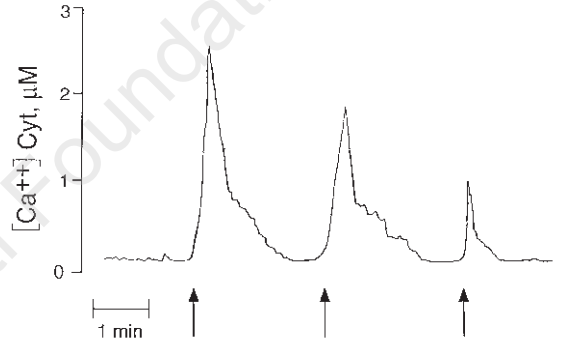

Figure 4. The addition of depolarizing concentration of KCl (50 mM) is followed by a short increase in cytoplasmic Ca^{++} levels, which progressively decreases after repeated additions.

ed channels on endothelial cell membrane.

As far as the effect of Ca^{++}-chelating agent EGTA on agonist-induced increase of cytoplasmic Ca^{++} levels in endothelial cells is concerned, it caused the complete suppression of Ca^{++} transients induced by KCl and by all the tested agonists but collagen, which was unaffected by EGTA addition (Table 1). These results suggest that collagen-induced increase in cytoplasmic Ca^{++} levels is related mainly to calcium mobilization from intracellular stores, while the increase induced by the other agonists is related almost exclusively to calcium influx from extracellular medium.

Effect of the calcium-channel blockers verapamil, flunarizine and nifedipine on agonists-induced Ca++ transients

Finally, the three calcium-channel blockers tested at the same final concentration (0.3 mM) did not significantly modify Ca^{++} transients, except for a reduction in thrombin-induced increase of cytoplasmic Ca^{++} levels caused by verapamil and flunarizine (but not nifedipine), which resulted statistically significant ($p < 0.05$). These results are summarized in Table 2.

Discussion

The vascular endothelium performs several important functions, vascular tone modulation, blood fluidity maintenance, protection against platelet-induced vasospasm and platelet aggregation, cytoprotection from free-radical-mediated polymorphonuclear cell damage, mechanoreception, etc., primarily by sensing changes in its environment and responding to these stimuli by producing a host of biologically active substances, known as endothelium-derived factors.[2]

Ca^{++} transients are critically important in the mod-

Table 2. Effect of different calcium channel blockers on agonist-induced increase of cytoplasmic Ca^{++} levels (μM) in endothelial cells (means±S.E. of 4 independent experiments).

Agonist	*Control*	*Verapamil 0.3 mM*	*Flunarizine 0.3 mM*	*Nifedipine 0.3 mM*
ADP (100 µM)	3.53±1.21	3.59±1.40	3.50±1.53	3.65±1.62
GRGDS (0.2 µM)	3.95±1.60	3.61±1.71	4.15±1.73	4.07±1.55
PAF (2 µg/mL)	2.32±.82	1.95±.75	2.07±.95	2.28±1.03
U46619 (20 µM)	2.35±.69	2.25±.71	2.28±.80	2.31±.90
Collagen (20 mg/mL)	3.93±1.35	4.05±1.86	3.91±1.79	3.82±1.63
Thrombin (10 U/mL)	6.40±1.79	3.81±1.44*	3.02±1.39*	5.95±1.93

$p < .05$ vs. control.

ulation of endothelium-dependant functions because many of the cytosolic enzymes responsible for the secretion of endothelium-derived factors are Ca^{++}-dependant.[11,23]

Since platelet agonists are well known inducers of Ca^{++} transients in platelets,[24] we tested the hypothesis that several platelet agonists and products of hemostatic system activation could also determine Ca^{++} transients in normal endothelial cells, which continuously interact with platelets in both normal and abnormal conditions.[2,25-29]

Our study demonstrated that platelet-agonists and products of the activation of the hemostatic system, stimulate endothelial cells causing Ca^{++} transients similarly to that observed in platelets and other non excitable cells, including tumor cells.[20]

Thus, ADP, GRGDS, PAF, TxA_2-mimetic U46619, collagen and thrombin were able to induce Ca^{++} fluxes in CPA-47 cells *in vitro*, while only PDGF was never able to induce Ca^{++} movements in this experimental setting.

Thrombin determined a different pattern of response when compared to the other agonists, with an initial steep increase in Ca^{++}, followed by a much more sustained elevation and slower return to the baseline. The stimulation of endothelial cells by repeated additions of ADP, GRGDS, PAF, U46619 and collagen was followed by Ca^{++} fluxes which resulted constantly stable in terms of duration and intensity, clearly showing that Ca^{++} transients were not exhausted by repeated additions of each agonist, with the remarkable exception of thrombin.

The different behavior observed after repeated thrombin stimulation, for example, the abrogation of response after the first stimulus, is probably due to the fact that shortly after thrombin receptor is activated, it is rapidly cleared from the cell surface.[30]

Thus, thrombin receptor, a member of the seven transmembrane domain receptor family,[31,32] is normally activated when thrombin binds to and cleaves the receptor's N-terminus making the previously occult tethered ligand domain available for interaction with still undetermined sites within the receptor remainder, but, after a brief burst of activity, the thrombin receptor shuts down possibly because of direct phosphorylation of the receptor itself. At approximately the same time, the activated receptor is internalized in response to an unidentified cue.[33,34]

Nevertheless, the endothelial cell itself is not exhausted after the first thrombin challenge, still being able to respond to the other agonists, as clearly shown by our experiments.

Among the effective inducers, GRGDS deserves a particular mention; to our knowledge, this is the first demonstration in which an RGD-containing peptide may trigger Ca^{++} transients in living cells *in vitro*. This observation is surely of interest: in fact, the RGD sequence is a cell-adhesion motif present in several matrix-associated adhesive glycoproteins, including fibrinogen, fibronectin and von Willebrand factor.[35] The RGD sequence is recognized by several integrins, mainly by $\alpha_{IIb}\beta_3$ (also designated GPIIb/IIIa), which are present on the membrane of a number of cells, including platelets, leukocytes, endothelial cells and cancer cells.[35,36] In platelets, GPIIb/IIIa is known to be implicated in activation induced by ADP and collagen and was found to act as a calcium channel.[37] Our data demonstrate that such a receptor may function as a calcium channel also in endothelial cells.

As far as calcium-channel blockers effect is concerned, they inhibit Ca^{++} transients through voltage-operated channels in excitable cells, but conflicting results have been published about their capacity to interfere also with receptor-operated channels.[20] In our study, only thrombin-induced Ca^{++} transients seem to be reduced by verapamil and flunarizine, but not nifedipine. This result is probably due to the difference in both structure and function of the different calcium-channel blockers used. Indeed, our results are preliminary and surely more extensive studies on the effects of different calcium-channel blockers are needed before any definitive conclusion can be drawn.

When the calcium chelant EGTA is added to the experimental system, ADP, GRGDS, PAF, U46619, and thrombin-induced Ca^{++} transients were abrogated, while collagen-induced Ca^{++} fluxes remained unaffected, thus suggesting the implication of different receptors and mechanisms, i.e., calcium influx from extracellular medium or calcium mobilization from intracellular stores,[20] as explained above.

The presence of voltage-operated channels also on endothelial cell membrane is supported by the results obtained with the addition of depolarizing concentrations of KCl to the experimental system; thus, short increases in cytoplasmic Ca^{++} levels, progressively decreasing after repeated additions, were evidenced in our study.

Even though many question remains unanswered, these results may help more thorough understanding

of the biochemical basis of the interaction between endothelium and platelets, a complex system with several, important implications for disease pathogenesis.

In particular, possible effects of endothelial cells adhesion and confluence in eventually modifying Ca^{++} transients within the experimental system we used, should be more extensively studied. Furthermore, human umbilical vein endothelial cells should also be investigated to prevent biases deriving from possible differential responses between freshly isolated human cells and a stabilized bovine aortic endothelial cell line.

Contributions and Acknowledgments

All three authors contributed equally to the study design, experimental conduction and paper writing.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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rHuEpo for the treatment of anemia in myelofibrosis with myeloid metaplasia. Experience in 6 patients and meta-analytical approach

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Abstract

Background and Objective. **Experience with recombinant human erythropoietin (rHuEPO) in the treatment of the anemia secondary to myelofibrosis with myeloid metaplasia (MMM) is slight up to now. We present our results of the treatment of 6 patients and a review of the literature in search of possible parameters predicting response to this treatment.**

Design and Methods. **From January 1994 to June 1996 all transfusion-dependent patients with MMM diagnosed in our hospital were included in this study. We established a minimum period of 4 weeks of treatment and a maximum of 12 if no response was observed. Initial dosages used were 100 U/kg s.c. 3 times weekly, increasing by 50 U/kg every 4 weeks where no response was observed. Response was defined as a reduction ≥ 30% of the previous transfusional needs. The review of the literature was made using a MEDLINE© search (January 1990-December 1996) on the keywords erythropoietin, myelofibrosis, and agnogenic myeloid metaplasia. A statistical study was made in search of possible parameters to predict response. The parameters studied include age, sex, hemoglobin, serum erythropoietin (sEPO) levels, transfusional dependency, transfusional requirements per month prior to treatment, maximum dosages used and dosage at which response was obtained.**

Results. **Only 2 of our 6 patients responded, both at a dosage of 600 U/kg/week (200 U/kg 3 times weekly s.c.). In addition to our 6 patients we have found only 28 other patients in the literature. For statistical calculation 2 of our patients were not considered as they did not complete the period of study. The overall rate of response was 17/32 (53.1%). In the univariate analysis comparing responders and non-responders we found a tendency to significance with respect to sex (p=0.07), sEPO (p=0.07) and transfusional needs in units of packed red blood cells per month (PRBC/m) (p=0.13). In this way patients with low sEPO, females and those with low transfusional needs (< 3 PRBC/m) respond better. This better response in females could be explained by the fact that their disease situation was more stable (with both lower sEPO levels and transfusional dependency). The best cut-off point in the sEPO to predict response was 123 mU/mL. No important side-effects have been observed except three cases of aggravation of splenomegaly. In two cases this condition improved when the rHuEPO was discontinued. The association of rHuEPO with hydroxyurea or interferon does not seem to affect the response.**

Interpretation and Conclusions. **Though the number of patients is low, our data suggest that some MMM patients, in particular females and individuals with low sEPO levels and with low transfusional needs, might benefit from rHuEPO in terms of elevation of hemoglobin levels. Unfortunately, transfusion dependent-patients, i.e. those in whom a beneficial effect of rHuEPO would be most welcome, are unlikely to respond, and more generally, treatment is not cost effective in medically responsive patients.**

Key words: erythropoietin, myeloproliferative disorders, myelofibrosis, agnogenic myeloid metaplasia, predictive factors

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Recombinant human erythropoietin (rHuEPO) has been used with moderate success in the treatment of anemia secondary to some pathologies involving the stem cell, such as myelodysplastic syndromes (MDS),[1] chronic myelogenous leukemia[2] or paroxysmal nocturnal hemoglobinuria.[3] Myelofibrosis with myeloid metaplasia (MMM) is a process affecting the stem cell that frequently produces anemia as well. In its treatment, among other drugs, androgens[4] or hydroxyurea (HU)[5] have occasionally been used but supportive therapy remains essential to maintain an acceptable quality of life in these patients. The use of rHuEPO in MMM has been very limited and experience with this drug is still slight. We present our results of the treatment of 6 patients with MMM, three of whom have been previously reported as showing no response.[6] A review of the literature has also been made in order to establish the possible parameters that could help to select those patients who could benefit from this therapy.

Materials and Methods

Patients

From January 1994 to June 1996 all transfusion dependent patients with MMM diagnosed in our Hospital were included in this study. Criteria for diagnosis included marrow fibrosis, splenomegaly and a leukoerythroblastic blood picture.[7] The trial could not be approved by the Ethical Committee of our Institution as the Committee did not exist at the beginning of the trial (it was formed in March 1996). However, informed consent was obtained from all patients. Transfusion dependency was the only criterion considered for inclusion in the study. From each patient data on age, sex, period of time since diagnosis, transfusional period, and other concomitant treatments were compiled as well as data regarding basal (prior to rHuEPO treatment) hemoglobin (Hb), leukocytes, platelets, reticulocytes, erythroblasts, ferritin, basal serum erythropoietin (sEPO), degree of transfusional dependency in units of packed red blood cell per month (PRBC/m) and size of the spleen before and after treatment. Basal Hb was calculated as the mean of the Hb levels before transfusion in the three months prior to treatment. PRBC/m was calculated as the mean of the transfusion requirements in the same period of time. Hb and PRBC/m post-treatment were obtained as the mean of their values after the beginning of treatment. Adverse effects during the period of study were also recorded if present. We established a minimum period of 4 weeks of treatment and a maximum of 12 if no response was observed. Initial dosages used were 100 U/kg s.c. 3 times weekly, increasing by 50 U/kg every 4 weeks if there was no response. This was defined as a reduction of at least 30% of the previous transfusional needs.

Selection of papers

The review of the literature was made using a MEDLINE© search (January 1990-December 1996) on the keywords erythropoietin, myelofibrosis, and agnogenic myeloid metaplasia. Those papers found were used as a source of new papers to include more patients (Table 1). We accepted the response criteria indicated in each study since differences were slight. These response criteria were variable, and in one study several could be considered, but they can be divided into 2 groups: ≥ 1 g/dL increase in the Hb levels, and ≥ 30% reduction in the degree of transfusion dependency. In order to compare the dosages used all were transformed into units/kg/week; if the data referring to sex, age, Hb, PRBC/m, sEPO, and dosage at which response was obtained were not clearly expressed they were not considered in the statistical analysis.

Statistical methods

Continuous parameters were described in terms of mean values (range). Categories were compared with Fisher's exact test and quantitative parameters with the Mann-Whitney U-test. ROC curves were used to calculate the best sEPO cut-off point to predict response. The expression "*n=*" indicates the number of patients available for each calculation.

Table 1. Articles included in the review of the literature. Results and maximum dosages used.

Year	*Authors*	*Response/ patients*	*Dosage° (U/kg/week)*
1990	Frutchman et al.	2/2	300 s.c.
1991	Iki et al.	0/1	15,000 i.v.*
1992	Cazzola et al.	1/3	750 s.c.
1992	Mittelman et al.	1/1	Not indicated s.c.
1992	Rafanelli et al.	1/1	1,050 s.c.*
1993	Aloe Spiriti et al.	4/7	800 s.c.
1993	Mohr et al.	0/2	1,680 s.c.
1993	Ziegler et al.	1/1	1,500 i.v.
1994	Tefferi et al.	0/4	450-900 s.c.
1994	Falkson et al.	2/3	Not indicated s.c.
1996	Bourantas et al.	3/3	600 s.c.*

*°Maximum dosage used; *fixed rHuEPO doses.*

Results

Results in our patients

From January 1994 to June 1996, six patients were included in the study. Table 2 shows epidemiological data of the patients as well as clinical and analytical parameters pre- and post-treatment with rHuEPO. Patient #1 was in the terminal stage and HU (500 mg every other day) was used during the last month of treatment together with rHuEPO; initial dosage was not modified due to his clinical situation and he died 2 months after beginning treatment due to progression of his disease. Patient #2 was initially considered as unclassified MDS (because no splenomegaly and a clear leukoerythroblastic blood picture was observed) evolving to MMM with progressive anemia. This case was reviewed later (prior to treatment) and the possibility of MDS was ruled out. He died 5 weeks after beginning treatment due to cardiac failure (this patient had previously suffered cardiopathy) but a rapid progression of the disease was observed during this period. Patient #3, in the terminal stage (as was patient #1) began HU (500 mg every other day) one month before beginning treatment with rHuEPO and this was maintained during the entire period of treatment. Patient #4 was treated for 9 months with 200 U/kg without requiring transfusional support; after this period she became newly transfusion-dependent (2 PRBC/m, the same amount as prior to rHuEPO treatment) and treatment was discontinued. She died

Table 2. Epidemiological, clinical and analytical data of our patients pre- and post-treatment with rHuEPO.

	*Patient #1**	*Patient #2**	*Patient #3*	*Patient #4*	*Patient #5*	*Patient #6*
Sex	Male	Male	Male	Female	Female	Male
Age	67	80	57	80	73	70
Response	NO	NO	NO	YES	YES	NO
T. Diagnosis (months)	10	6	17	96	15	2
T. Transf. dep. (months)	8	1	17	2	15	2
Initial dosage (U/kg)	100	100	100	100	100	100
Maximum dosage (U/kg)	100	150	200	200	200	200
Hb (g/dL)	8.9/10.6	10.5/9.3	6.7/6.8	8.7/9.9	9.7/10.3	9.1/9.7
Transfusion (PRBC/m)	8.3/9.5	3.6/8	10/10	2/0	2/1	7/6
sEPO (mU/mL)	17/56	26/NA	826/1507	16/4	123/233	510/512
Reticulocytes (%)	0.5/0.1	3.6/0.4	1.3/0.8	2.2/3	1.3/1.4	0.6/0.1
Erythroblasts^	1/0	2/0	5/4	6/21	2/0	0/0
Ferritin (ng/mL)	1620/2430	272/NA	2780/2850	738/1740	2230/1380	738/1840
Leukocytes ($\times 10^9$/L)	5.8/6.4	13.4/11.9	35/15	9.9/7.9	6.4/7	13.4/14.7
Platelets ($\times 10^9$/L)	333/291	109/204	20/15	164/181	189/231	222/216
Splenomegaly (cm)	15/25	5/5	10/12	9/9	3/0	5/8
Treatment (weeks)	8	5	12	12	12	12
Other treatment	HU	No	HU	No	No	No

*Patients #1, 2 and 3 were previously reported (ref. #6). *Not included in statistical study as the period of study was not completed; T. Diagnosis = Time since diagnosis; T. Transf. dep.= Time with transfusional dependency; Hb= Hemoglobin; PRBC/m = units of packed red blood cells/month; sEPO = serum erythropoietin; ^Over 100 leukocytes; HU= hydroxyurea.*

2 months later at home (cause unknown). In patient #5 treatment was maintained at a dosage of 200 U/kg after the period of study as her transfusional needs were reduced by 50% in this period and she became transfusion-independent one month later. She is still on treatment (subsequent follow up 6 months without transfusions). Patient #6 did not show any response to treatment and this was discontinued.

No effect on the other hematological series was observed except in patient #2 in whom the platelet count doubled. Only in patient #1 was an aggravation of the splenomegaly observed. No other adverse effects due to the treatment were observed during administration.

Results from the literature and our patients

A total of 28 other patients was compiled from 11 articles.[8-18] We made a statistical analysis of the data from all the patients. Data from 32 patients were analyzed. Patients #1 and #2 in our series were excluded as they did not complete the period of study proposed. With respect to sex (n=24) 16 were males and 8 females, mean age (n=29) 64.3 years (22-80). Mean values of the parameters analyzed were: basal Hb (n=24) 8.1 g/dL (5.4-12); basal sEPO (n=15) 573.5 mU/mL (7.9-2,281); seven patients did not require transfusions and of those who were transfusion-dependent, transfusional requirements were (n=15) 4.2 PRBC/m (2-12); maximum dosage used (n=24) 1,377.5 U/kg/week (300-15,000); dosage at which response was obtained (n=14) 721.4 U/kg/week (300-1,500). The response rate was 17/32 patients (53.1%). In a univariate analysis we found no significant differences ($p>0.05$) with respect to any of the parameters considered. However, differences approached statistical significance with respect to sex ($p=0.07$), sEPO ($p=0.07$) and PRBC/m ($p=0.13$). Table 3 shows the differences between responders and non-responders and their statistical significances. One consideration can also be made; if we exclude, from among the responders, the only heavily transfused patient differences with respect to PRBC/m become significant ($p=0.04$). All but one responding patient had a basal sEPO $\leq$ 123 mU/mL, and this was the best cut-off point found (sensitivity 0.86; specificity 0.87). Multivariate analysis could not be performed as the number of patients was low and not all the data were available.

Ten of the twenty patients who were transfusion-dependent become transfusion-independent and six of the seven non-transfusion-dependent patients responded. The duration of response is known in 7 patients (in months): 10+, 16+, 7, 6, 9, 9, 6+ respectively. The only effect on other hematological series concerns platelets which decreased in 2 patients and increased in another two. However, four studies do not provide any information on this point. In 3 patients (n=14) aggravation of splenomegaly was observed and in two of these the size of the spleen decreased after reducing or suspending treatment; in

Table 3. Comparison between responding and non-responding patients. Statistical significance in the univariate analysis.

	Responders	*Non-responders*	*p**
Sex (M/F)	7/7	9/1	0.07
Age (years)	66.2 (SD±8.2)	62.3 (SD±15.3)	0.77
Basal Hb (g/dL)	8.2 (SD±1.6)	8.0 (SD±0.9)	0.77
Basal sEPO (mU/mL)	175.1 (SD±325.4)	922.0 (SD±841.2)	0.07
Transf. Dep (PRBC/mL)	2.1 (SD±3.0)	4.4 (SD±3.4)	0.13
Dosage (U/Kg/week)	721.4 (SD±299.8)	2296.0 (SD±4486.9)	0.26

**Significance limit p<0.05; M/F = male/female; sEPO = serum erythropoietin; Trans. dep (PRBC/m) = transfusional dependency (units of packed red blood cells/month); dosage = maximum dosage used.*

another 4 a reduction of the splenomegaly was observed. Three of these were treated simultaneously with interferon. Five patients (n=29), three of whom responded, received simultaneous HU. Another three received interferon (IFN) simultaneously: all responded. No adverse effects have been reported; one patient died due to myocardial infarction and in another, evolution to acute myeloid leukemia was observed.

Discussion

Anemia is a consistent finding in MMM. Though the origin of this myeloproliferative disorder is unknown, several factors have been considered as possible causes of anemia: ineffective erythropoiesis,[16] inappropriate erythropoietin response to anemia[10] and bone marrow fibrosis.[13] Recently, Barosi *et al.*[19] reported that 87% of patients with MMM presented with elevated levels of sEPO according to their degree of anemia and postulate that anemia is not due to an inappropriate secretion of erythropoietin but to a defective response to it. Some of these causes are shared by other pathologies derived from stem cell disorders such as MDS in which rHuEPO has been widely used with moderate success[20,21] in comparison with other pathologies in which levels of sEPO are considered *low* such as renal dialysis patients,[22] HIV-infected patients in treatment with AZT[23] or anemic cancer patients.[24] The treatment of anemia in MMM has long been mainly transfusional, but the developement of rHuEPO has theoretically provided a new tool. Though Barosi *et al.*[19] believed that no beneficial effect could be expected from this treatment in those patients with high endogenous levels of sEPO and Eschbach *et al.*[22] reported that those patients with end-stage renal disease who presented with myelofibrosis did not respond to rHuEPO, several authors have used it in the treatment of anemia in MMM (Table 1).

Attention must be drawn to several aspects of the statistical analysis: a) it seems that patients with lower transfusional requirements respond better (differences did not reach statistical significance, p=0.13). In fact, six of the seven transfusion-independent patients showed response and the rest of the responders but one received ≤ 3 PRBC/m, while all but three of the non-responders received more than this quantity. If we exclude the only heavily transfused responder patient differences reach statistical significance (p=0.04); b) patients with lower levels of sEPO respond better than those with higher levels (differences are almost significant, p=0.07). From these data a cut-off point of 123 mU/mL of sEPO was the most effective to identify those patients with a higher possibility of being responders; all patients but one with sEPO levels above this were not responders. This cut-off point is lower than that previously reported by our group (250 mU/mL)[25] or by Hellström-Lindberg (200 mU/mL)[20] in patients with myelodysplastic syndromes but is slightly higher than that reported by Rose *et al.*[21] or Cazzola *et al.*[26] (in both cases 100 mU/mL) in patients with the same diagnosis. Differences with respect to sex nearly reached statistical significance (p=0.07) and could be explained by the fact that females had lower levels of sEPO and transfusional needs. However, all these conclusions must be taken with caution as the number of patients in the analysis was small. In overall terms our results suggest that the patients with the best medullary or extramedullary functional reserves, those with lower transfusional requirements and those with lower sEPO levels as well respond better than others. In our experience, patients in the terminal stage of the disease do not benefit from rHuEPO treatment.

It is interesting to point out that though in dogs rHuEPO has been found to produce myelofibrosis this has not been observed in hemodialyzed patients.[27] We have found no data regarding this circumstance except in one patient in whom fibrosis did not progress after 9 months of treatment,[15] but it would be interesting to study more patients to observe whether the degree of fibrosis progresses more rapidly in these patients than in others not treated with rHuEPO.

A question still to be answered is whether the concomitant use of HU could improve the results obtained using rHuEPO alone. This combination has been used successfully to improve anemia in beta-thalassemic patients,[28] and it is known that HU has been used in patients with MMM to reduce splenomegaly, leukocytosis or thrombocytosis and to increase Hb as well as to improve their clinical status with the advantage that the leukemogenic effect is minimal.[29,30] In our experience, those patients treated with HU (patient #1, who did not finish the study, and patient #3) did not respond although another 4 patients in the literature received this treatment and,

of these, three responded, so, in overall terms, 60% of patients treated simultaneously with HU showed response. All the three patients who received simultaneous IFN responded. However, the authors of that report[18] believe that the response could have been due to the fact that all these patients were in the proliferative phase of the disease. In the same report another 4 patients were treated with a combination of GM-CSF, rHuEPO and IFN. All responded. This is the only article reporting a combination of growth factors in the treatment of MMM.

Splenomegaly is another consistent finding in MMM and is a consequence of the extramedullary foci of hemopoiesis. In our patients aggravation of splenomegaly while being treated with rHuEPO was observed in patient #1 but we believe this to be due mainly to progression of his disease. However, 2 cases have been reported,[9,10] in which this increase of the splenic size was observed and in both the size of the spleen reduced after discontinuing treatment. The concomitant use of HU or IFN could perhaps avoid this enlargement of the spleen. In one of our responding patients, splenomegaly disappeared with rHuEPO treatment. This effect has been previously reported in four patients.

Effects of rHuEPO on the other hematological series seem to occur rarely and have affected only platelets. In one of our patients the platelet count almost doubled. In the literature, another patient experienced an increase, while two suffered a decrease in their platelet counts. The evolution of MMM to acute myeloid leukemia is well known, but the only episode registered in these patients was observed, not while receiving treatment, but 7 months later.

In conclusion, rHuEPO can be an effective treatment for anemia in patients with MMM, especially in those with low sEPO (≤ 123 mU/mL) levels and low transfusional dependency (≤ 3 PRBC/m). Female sex was also associated with a better response. However this could be due to a more stable situation in their disease (with both lower sEPO levels and lower transfusional dependency). However, due to the high cost of treatment we believe that this should be considered in the light of the life expectancy of patients and their quality of life. In addition, our conclusions are based on the results obtained in a few patients and further studies with a greater number of patients are required for confirmation.

Contributions and Acknowledgments

DP and JNR were responsible for the design of the study. MLM, JNR and JCD contributed to the execution of the study. All the authors contributed to the analysis and writing of the paper. Though all authors have contributed substantially in the article, the authors are ordered according to their contribution in the final form of the article.

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Disclosures

Conflict of interest: none.

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Manuscript processing

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Cyclosporin-A in severe refractory anemia of myelofibrosis with myeloid metaplasia: a preliminary report

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Abstract

Background and Objective. **Severe anemia is the outstanding problem in approximately 50 percent of patients with myelofibrosis with myeloid metaplasia (MMM). The present trial was based on the considerations that abnormal immune responses are frequently associated with MMM and that cyclosporin A (Cy-A) has proven to be effective in improving anemia in autoimmune disorders. The aim of this study was to evaluate the effect of Cy-A on anemia of MMM.**

Design and methods. **We studied 10 patients with MMM and severe anemia who were not responsive to corticosteroids. Eight of them showed evidence of immune defects (direct or indirect Coombs' test, antinuclear or antimitochondrial antibodies, circulating immune complexes). Cy-A was delivered orally in two refracted doses of 5 mg per kilogram bw every day and the serum level of the drug was maintained between 100 and 200 ng/mL for at least 6 months. Clinical effects were measured by calculating a normalized transfusional need (NTN), and response was defined as about a 30% reduction in the initial transfusion requirement. Hematologic parameters, s-Epo, s-TfR, s-IL2R and lymphocyte flow cytometric analysis were also evaluated. The results were analyzed with the Student's t-test.**

Results. **Only 6 patients completed the entire 6 months of planned therapy. Three of these responded, with one no longer needing transfusions. A high CD4/CD8 ratio was predictive of response (mean value 4.7±3.5 in responders versus 0.9±0.4 in non-responders, p=0.06).**

Interpretation and Conclusions. **An immunomediated mechanism negatively affects erythropoiesis in MMM. Cy-A may be effective for patients with severe refractory anemia in this disease.**

Key words: cyclosporin A, anemia, myelofibrosis with myeloid metaplasia, immune disorders, CD4/CD8 ratio

Myelofibrosis with myeloid metaplasia (MMM) is a chronic myeloproliferative disorder with a heterogeneous clinical picture. Approximately 50% of patients have severe anemia at diagnosis or during the evolution of the disease.[1] First line treatment with corticosteroids and androgens is effective in one-third to half of all cases;[2] thus red blood cell (RBC) transfusions are frequently needed. The present study tested the efficacy of cyclosporin A (Cy-A) in treating the anemia of patients with MMM who were refractory to first line treatments. The rationale for this study stems from the considerations that Cy-A selectively inhibits immune responses mediated by T lymphocytes and has proven to be effective in improving anemia in disorders sustained by autoimmune mechanisms such as aplastic anemia,[3] pure red cell aplasia,[4] and immune hemolytic anemia.[5] Disruption of the immune response with the development of Coombs'-positive autoimmune hemolytic anemia, nephrotic syndrome, antinuclear antibodies, rheumatoid factor, lupus-type anticoagulant and hypocomplementemia have all been documented in MMM.[6-12] T lymphocyte activation has also been reported and the poor prognostic significance of elevated soluble IL-2 receptor has been evidenced.[13] These alterations suggest either clonal involvement of the lymphocyte population in the myeloproliferative disorder or secondary activation of the immune system due to abnormal monocyte-macrophage function. Another reason for this study was that patients responding to Cy-A have recently been described in other clonal myeloid disorders like myelodysplastic syndromes.[14,15]

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Materials and Methods

Subjects

Ten patients with MMM and severe anemia were evaluated according to a protocol approved by the hospital's ethical committee. MMM was diagnosed on the basis of bone marrow biopsy demonstrating moderate to severe bone marrow fibrosis, peripheral blood morphology showing immature erythroid and myeloid cells, tear drop erythrocytes, absence of absolute monocytosis, and splenomegaly. Myeloid metaplasia was documented by ferrokinetics in one patient, and by histological examination of the removed spleens in three others.

Patients were eligible to enter the study if they suffered from transfusion-dependent or pending-transfusion anemia, and showed no response to corticosteroid therapy (equivalent to a daily dose of 25 mg of

prednisone) after 3 or more months of treatment. All patients had received other therapies, including androgens, 1-25,dihydroxyvitamin D3 or danazol. Three of them had been splenectomized from 1 month to three years earlier. None of the participants showed abnormal renal function (BUN and serum creatinine were normal) upon entry to the study.

Treatment

The patients were scheduled to receive a six-month course of Cy-A, 5 mg per kilogram per day orally. This dosage is recommended as a higher maintenance dose in most autoimmune diseases and was used in our study as an attack dosage. Cy-A was gradually tapered both to maintain plasma levels between 100 and 200 ng/dL and on the basis of renal function tests. The dose was reduced if the serum creatinine concentration increased 30% over baseline. The lower dosage was 3 mg per kilogram per day. Patients receiving corticosteroids at entry continued to receive them.

The primary endpoint of the study was improvement of anemia. Even though the general policy was to transfuse RBC when Hb levels dropped below 80 g/L, different transfusional regimens were active during the study. In order to compare various regimens among patients and at different periods of time, a normalized transfusional need (NTN) was calculated, defined as the number of RBC units theoretically needed to maintain the Hb value equal to 100 g/L. By assuming a linear relationship between the blood supplied and the Hb concentration actually maintained, the following proportion was used:

N. of RBC units given in one month : normalized mean Hb (g/L) maintained during the month = NTN : 100

The normalized mean Hb value maintained in the monthly period was calculated by the area under the curve of all the Hb concentration values measured during that month. Patients whose NTN did not fall by 30% of the basal value for 2 consecutive measurements were considered not to have responded to the treatment. Biochemical studies, including tests of renal and hepatic function, were performed every month.

Assessment of erythropoiesis

Hematologic parameters, serum erythropoietin (s-Epo) and serum transferrin receptor (s-TfR) were tested at the time of enrolment and every month during treatment. Hemoglobin concentration, PCV, red blood cell count, erythrocyte indices and reticulocyte count were obtained with an automatic cell counter (ELT-800, Ortho Diagnostic System, Massachusetts, USA). S-Epo and s-TfR levels were determined using venous blood sampled without anticoagulant. Serum was separated by centrifugation within 12 hours of collection and stored at –20°C until thawed for assay. An enzyme-linked immunosorbent assay (CLINIGEN, Amgen Diagnostic, Thousand Oaks, CA, USA) was employed. Intra- and inter-assay variation coefficients differed at the different sample concentrations but were always less than 10%.

Assessment of immune function

Tests for antinuclear antibodies (ANA, ENA, anti-dsDNA), antimitochondrial and anti-smooth muscle antibodies (AMA, ASMA), direct and indirect Coombs' and circulating immune complexes (CIC) were performed at the time of enrolment and on completion of treatment.

The serum interleukin-2 receptor (s-IL-2R) was assayed as a marker of T-lymphocyte activation using an enzyme immunoassay kit (T Cell Diagnostics, Inc. Woburn, MA, USA). Peripheral blood mononuclear cells were examined by flow cytometry analysis at the time of enrolment and every month during treatment. One hundred mL of whole blood were incubated with monoclonal antibodies (MoAbs) conjugated with FITC or PE and harvested with a Multi-Q-Prep technique (Coulter Ltd, Hialeah, FL, USA). Flow cytometry (two-color) was performed with an EPICS XL-MCL (Coulter, Ltd). T and B cells were identified by MoAbs against CD2, CD3, CD4, CD8, CD19 and CD20, and activated cells were detected by MoAbs HLA-DR and CD25, while NK cells were identified by CD56. All MoAbs were purchased from Coulter except CD56, which was obtained from Becton Dickinson (Mountain View, CA, USA).

Statistical analysis

Quantitative variables were compared with the two-tailed Student's t-test. A p value less than 0.05 was considered significant.

Results

Clinical and hematologic features

Mean age, duration of disease, duration of corticosteroid therapy before the study, the number of patients requiring blood transfusions and the number of those requiring concomitant medications before and during the trial are reported in Table 1. The hematologic parameters are also described in the same table. Besides anemia, 7 patients also presented leuko- or thrombocytopenia. Bone marrow biopsy documented a normocellular picture in one, hypocellular in 8, and selective erythroid aplasia in 1 patient.

Immunologic features

Laboratory signs of immunodysfunction were found in 8 patients: documented by a positive indirect Coombs' test in 3 patients who had been repeatedly transfused, by a direct Coombs' test in one, an ANA titer of 1:160 with a diffuse pattern in 2, an AMA titer of 1:40 in one and CIC in 2.

Flow cytometry analysis revealed that the percentage of CD4 cells was below the normal range in 1

Table 1. Baseline characteristics of the 10 study patients with MMM.

Mean age – yrs (range)	60.9 (42–72)
Sex – M/F	6/4
Mean duration of disease – months (range)	36.4 (3–147)
Concomitant corticosteroid therapy before and during the trial – no. of patients (%)	4 (40)
Mean duration of corticosteroid therapy before the study – days (range)	122 (20–540)
Transfusions – no. of patients (%)	8 (80)
Hemoglobin concentration at the beginning of the trial – mean (range) g/L	72 (48–95)
White blood cells count at the beginning of the trial – mean (range) $\times 10^9$/L	6.8 (1–24)
Platelet count at the beginning of the trial mean (range) $\times 10^9$/L	86.6 (9–236)
Spleen volume – no. of patients	
splenectomized	3
above the umbilical line	4
below the umbilical line	3
Bone marrow biopsy – no. of patients	
normocellular	1
hypocellular	8
erythroid aplasia	1

out of 8 patients and the percentage of CD8 cells was above normal in 5 out of 8, with low CD4 absolute counts in 3 patients and high CD8 counts in 2. Four patients had an increased percentage of CD3/HLA-DR-positive cells, indicating activation of cytotoxic T cells (Figure 1). The CD4/CD8 ratio was higher than normal in 2 out of 8 patients (3.2 and 8.8, respectively; normal values ranged from 1.3 to 2.9). Two of the 3 splenectomized patients showed the lowest values (respectively, 0.5 and 1). S-IL-2R was increased in 6 out of 8 patients (from 1103 to 6641 U/mL) with respect to the values detected in normal healthy subjects (range 237-943 U/mL).

Status of erythropoiesis

Reticulocyte count was normal or below normal in all but 3 patients, who presented with mild reticulocytosis (396, 108 and 127×10^9/L, respectively). One of these also had decreased serum haptoglobin, mildly increased nonconjugated bilirubin (1.8 mg/dL) and a positive indirect Coombs' test. S-Epo ranged from 3.6 to 5031 mU/mL (reference range from 5 to 20 mU/mL). The ratio between observed and predicted values with respect to anemia[16] was lower than 0.8 in one case, indicating that Epo production was inadequate for the degree of anemia. S-TfR ranged from 172 to 6818 ng/mL (normal reference values from 1470 to 3400 ng/mL). Only in one patient, who also presented a histological pattern of bone marrow hypoplasia, was the value lower than normal, documenting reduced erythropoiesis.

Response to cyclosporin treatment

Six of the 10 patients concluded the 6-month treatment schedule. One patient interrupted the drug treatment because of evolution toward blast transformation, 2 others did not comply with treatment and one developed progressive renal failure. Three of the 6 evaluable patients responded to treatment. One of these obtained complete independence from RBC transfusions. Before treatment he needed 6 units of RBC every month to maintain a mean pre-transfusional Hb level of 62 g/L. At the end of treatment his mean Hb concentration remained at 92 g/L without transfusions. Two other patients reduced their NTN from 2.9 and 1.8 units of blood/month to 1.2 and 1.1, respectively. The improvement was slow in all patients: the reduction in transfusional need became apparent within 4 months after treatment was begun,

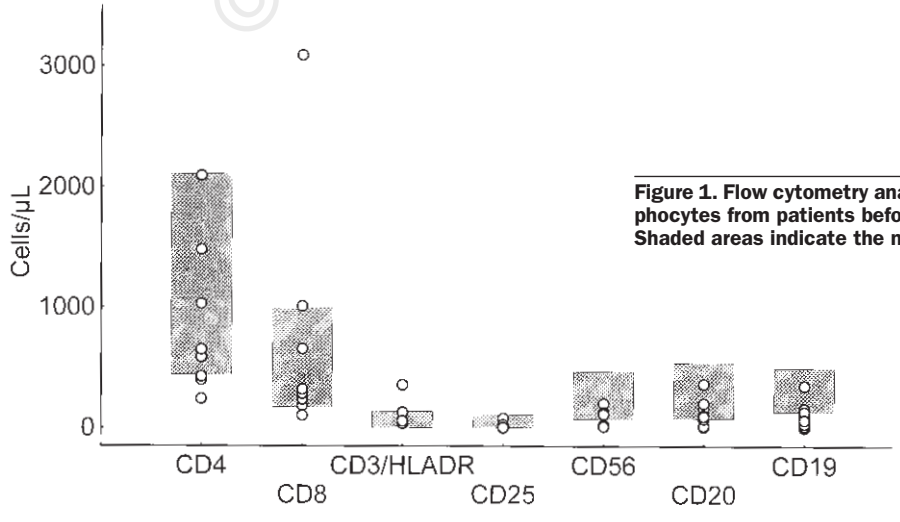

Figure 1. Flow cytometry analysis of peripheral blood lymphocytes from patients before treatment with Cy-A. Shaded areas indicate the normal range.

and the optimally responding patient was transfusion-independent at the sixth month.

During treatment, the reticulocyte count decreased in one patient who responded to therapy (from 77 to 49×10^9/L) and in another who did not respond (from 108 to 16×10^9/L). In both these patients s-TfR also declined: from 2242 to 1407 ng/mL in the former, and from 1679 to 834 ng/mL in the latter; however, a drop in sTfR (from 3,932 to 2,388 ng/mL) was also noted in a case that had no change in the reticulocyte count. No changes in the haptoglobin level were documented. S-Epo decreased in all patients during therapy: from a mean value of 1,491 mU/mL, range 105-5,032, to a mean value of 557 mU/mL, range 95-1,609 ($p<0.01$).

A decrease in the CD4/CD8 ratio was observed in 3 of the 6 evaluable patients. This was due to a decline in the CD4 subset of lymphocytes. At the end of the sixth months of therapy, an indirect Coombs' test was found to be negative in 2 of the 3 previously positive patients. ANA and AMA tests were negative in all patients. No changes in leukocyte or platelet counts were detected. IL2R decreased in one patient who did not respond (from 6,641 to 4,070 U/mL).

Three patients continued to receive the drug for periods ranging from 6 to 12 months after the conclusion of the study and they maintained the hematologic response. Thereafter there was a progressive increase in their transfusional need and therapy was subsequently stopped.

Predictors of response

Patients who responded to Cy-A therapy had the most severe transfusional need. NTN ranged from 1.8 to 2.9 (mean 2.4±0.5) in responders, while it varied from 0 to 2 (mean 0.9±1, $p=0.04$) in nonresponders. All the responders had an intact spleen and a positive indirect Coombs' test. The characteristic that best divided the patients who subsequently responded to Cy-A was the CD4/CD8 ratio, which showed a mean value of 4.7±3.5 (range 2.3 to 8.8) in responders and 0.9±0.4 (range 0.47 to 1.4) in nonresponders ($p=0.06$). There was no association between response to Cy-A and the previous or concomitant use of corticosteroids.

Plasma cyclosporin-A concentration

Among the 6 patients who concluded the treatment, the mean plasma Cy-A concentration was 124.5 ng/mL (range 59 to 185 ng/mL). There was no association between plasma Cy-A concentration and the rapidity of response.

Adverse effects

Four of the 10 patients treated with Cy-A suffered nephrotoxicity. Four experienced paresthesias. One patient developed gingival hyperplasia that improved after Cy-A was discontinued.

Discussion

The immunosuppressive properties of cyclosporin A were the reason for its use in patients with MMM and severe anemia in this trial. A high frequency of immune defects has been described in MMM patients[6-12] and most of the resulting abnormalities are able to produce anemia. As a matter of fact, 70% of our patients had alloantibodies against transfused red cells, autoantibodies against red cells, antinuclear or antimitochondrial antibodies. This frequency of immunodysfunction was similar to that reported in the literature, which ranges from 10 to 80%.[6-12,17]

This preliminary report documents that Cy-A is not an easy therapy to administer in patients with MMM. Four of 10 participants did not complete the scheduled 6 months of treatment: one due to evolution towards the blast phase of the disease, 2 due to noncompliance and one because of toxic effects on renal function.

Three out of the 6 evaluable patients responded to therapy, and one of these experienced complete disappearance of a severe transfusional need. A similar complete remission has been recently reported in a single case by Pietrasanta *et al*.[19] Response was evaluated on the basis of an index that assessed the theoretical transfusional need required to maintain a Hb level of 100 g/L, which was useful for comparing patients with different transfusional schedules. These results seem to indicate that Cy-A is able to modify mechanisms that negatively affect erythropoiesis. Even though no significant lymphocytosis within the bone marrow sections had been reported in our patients, high levels of s-IL2R were reported in 6 out 8 patients studied. These serum concentrations did not change during Cy-A therapy and the basal levels were not different in responders and nonresponders. So, no conclusion can be drawn on the meaning of this immune response index in patients with MMM, on the mechanism underlying Cy-A action.

A study of red cell production parameters supports the hypothesis of a peripheral effect of an immunosuppressive agent. In fact, reticulocyte count and s-TfR decreased in 2 and 3 patients, respectively, during treatment, indicating a reduction of hemolysis rather than a direct effect on erythroid progenitors.

Very severe anemia and a high CD4/CD8 ratio were the features that best predicted response to therapy. An elevated CD4/CD8 ratio was an unexpected finding in our patients since a reduction of this parameter is reported in patients with high transfusional needs, and splenectomy is known to increase the CD8 count without a proven effect on the CD4/CD8 ratio.[20] This suggests that a more detailed characterization of bone marrow and peripheral blood lymphocyte population would be of interest in these patients.

Further studies involving more patients are needed to substantiate our findings and confirm the possibility of response prediction.