Haematologica
Journal of Hematology
volume 84 – number 5 – May 1999

Haematologica 1998; 84:385-389 editorial, comments and views

Viral etiology of human cancer: a historical perspective

The hypothesis according to which some human cancers might be caused by filterable microorganisms such as viruses is almost one hundred years old. It was indeed in 1903 that Borrel, in France, suggested such a possible relationship. To put this hypothesis in a historical perspective one should refer to the book *The Riddle of Cancer*[1] which Charles Oberling published in 1952 and in which the possible role of viruses in human cancer was presented with extensive references to contributions of initial pioneers such as Rous, Shope, and Bittner. Since our purpose is, to some extent, focused on the evolution of methodologies which cancer researchers utilized in attempts to verify the hypothesis, one should emphasize that the approach of Rous and his followers was essentially based on establishing the difference between transmission of tumors and leukemias by cell transplants, i.e. grafts, or by cell-free filtrates. Transmission of tumors in experimental animals by cell-free filtrates was always interpreted as demonstration of viral etiology.

During the past fifty years, viral oncology has been studied in almost all cancer research centers, world wide. Practical results in terms of effective therapy of human malignancies have been nil. But still, recent issues of all the main oncology publications contain numbers of studies related to viral oncology, clearly indicating that the hypothesis still has considerable momentum!

The past fifty years can be analyzed in two distinct periods. The first, between 1945-1970 was dominated by electron microscopy; the second, from 1970 until now, being dominated by molecular biology.

Electron microscopy (EM) contributed a considerable amount of data, which can briefly be summarized as follows.

1. EM can readily demonstrate associations between viruses and cancers in several laboratory animal species, such as chickens and mice;[2,3]
2. EM data by themselves do not, however, prove any role of these viruses in the etiology of the tumors.[4] The EM data did, however, trigger microbiological experiments, based on ultrafiltration, which frequently yielded scientific evidence for etiological relationships;
3. as pointed out by André Lwoff *et al.*[5] in 1962, electron microscopy is probably the most efficient approach to viral classification;
4. viruses shown to be associated with several cancers of laboratory animals belong to various families of viruses (herpes, vaccinia, papova, retroviruses, DNA, RNA,...) and are not restricted to any one family;
5. viruses associated with some cancers and those responsible for infectious diseases look identical. There is no such thing as a family of oncogenic viruses, a terminology which never appears in general classifications of viruses and should actually be regarded as a misnomer;
6. practically, EM is essential to monitor the level of success in the sequential steps leading to virus isolation and purification. Therefore, the success of biochemical characterization of viral markers depends on electron microscopy to ascertain the purity of viral isolates and the absence (or minimal amounts) of non-viral contaminants;
7. finding particles with typical viral morphology does not mean that these viruses are pathogenic. Actually, there are probably many more non-pathogenic than pathogenic viruses. This point was well illustrated in a special conference sponsored around 1960 by the New York Academy of Sciences under the title *Viruses in Search of Disease*;
8. viruses, infectious or cancer-associated, rarely satisfy all the Koch postulates which, incidentally, were presented before viruses were discovered;
9. while the association between viruses and numerous malignancies of laboratory animals has been readily demonstrated by electron microscopy, and in spite of considerable efforts, similar associations have never been observed in human cancers[4] (with very rare exceptions such as the common wart and *molluscum contagiosum*...)

Of mice and men...

Research in viral oncology changed drastically around 1970, when methods of molecular biology took the lead, while electron microscopy was relegated to a distant background.

Dedicated *virus hunters*, as Peter Duesberg[6] would call them, were obviously not discouraged by the negative results of twenty years of active search for viruses by electron microscopy in many types of human cancers. To the contrary, large research programs were initiated, based primarily on the identification of viral, molecular *markers* such as enzymes, nucleic

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acids or proteins identified most frequently in cell cultures derived from human malignancies, rarely directly from the tumor tissues or blood plasma. The fact that viruses had never been directly observed in human tumors by electron microscopy was conveniently explained in terms of virus latency, and/or by integration of a provirus in the genome of tumor cells.

The most significant example illustrating this drastic change in approach is given by the reverse transcriptase enzyme, discovered in 1970 by Temin in purified Rous sarcoma virus,[7] and by Baltimore in Rauscher mouse leukemia virus.[8] This discovery was regarded as historical. It resulted in two Nobel prizes and in the renaming of all RNA tumor viruses as *retroviruses*. DNA synthesis from an RNA template was indeed a very surprising observation in 1970. The enzyme was initially thought to represent a unique feature of RNA tumor viruses and was, therefore, regarded as a reliable *marker* of the presence of *retroviruses*, even when retrovirus particles were never convincingly observed by EM. We learned, later on, that reverse transcription is a common phenomenon, that the enzyme (RT) is present in many different cells,[9] and that demonstration of RT activity is far from enough to substantiate any claim for the isolation of a *retrovirus*.

An exhaustive review of viral oncology literature over the past 25 years would be beyond the purpose of this editorial. Instead, it may be appropriate to mention publications which have appeared over the past few years, in an attempt to evaluate how much progress has been made toward the consolidation of a century old hypothesis.

Two main areas of viral oncology, namely: herpes viruses with emphasis on EBV and herpes-8 virus, and papova viruses with emphasis on human papilloma virus, HPV, will be considered. Retroviruses, although they have been the topic of my own research, will not be specifically emphasized here because the currently used anti-HIV antibody tests are non-specific (*vide infra*), making any attempt to analyze the current literature very hazardous.

Herpes

Abundant reports in oncology literature refer to the Epstein-Barr virus. This human herpes virus infects most people early in life. However, when primary infection is delayed until adolescence or adulthood it frequently causes infectious mononucleosis. It is a highly contagious agent (the *kissing disease*). Most EBV carriers are disease-free. However, in some cases EBV appears to be associated with several forms of cancers: lymphomas in immunosuppressed patients, Hodgkin's disease, Burkitt's lymphoma in central Africa, nasopharyngeal carcinoma, thymic lymphoepithelioma, primary nasal lymphomas and gastric carcinoma, of much more sizeable epidemiological importance. These malignancies, and their relatedness to EBV, have been recently reviewed in a 1998 Gann monograph edited by Toyoro Osato.[10] Several well characterized virus proteins (the EBNA proteins) are involved in the immortalization of B lymphocytes and, most interestingly, cyclin D2, driving B cells from the resting stage into G1, is induced within 24 hours of EBV infection.

Although B cells appear as the primary targets of EBV, T cells might also be involved as suggested by Kanegane *et al.*[11] in a study of patients with severe, chronic active EBV infection who came down with EBV-positive T-cell lymphoma.

Methods of *in situ* hybridization have been applied to the study of familial Hodgkin's disease by Lin *et al.*,[12] suggesting that EBV does not play an important role in familial Hodgkin's disease.

Studies at the University of Padova, by Ometto *et al.*,[13] support the notion that lymphomas arise from clonal expansion of EBV^+ cells. That latent EBV infection can be reactivated by EBV-specific $CD8^+$ T cells was demonstrated by Nazaruk *et al.*[14]

The danger of EBV-induced lymphoproliferative disease after allogeneic stem cell transplantation was studied, using a semiquantitative EBV-PCR technology, by Lucas *et al.*[15] Recently, Hale and Waldmann[16] analyzed the occurrence of EBV driven lymphoproliferative disorders in patients receiving T-cell depleted allogeneic bone marrow transplantation. They suggested that additional depletion of B cells is beneficial *possibly because it reduces the virus load or the virus target* which is hardly compatible with the limited success experienced with antiviral agents in such cases.

In America and in Europe, 50% of the cases of Hodgkin's disease are associated with EBV. According to Roskrow *et al.*,[17] EBV-specific cytotoxic T lymphocytes generated from normal donors may persist long-term *in vivo* and reconstitute the immune response to EBV, this possibly being an effective prophylaxis and treatment of immunoblastic lymphoma. The approach could be useful for cases failing to respond to salvage chemotherapy.

The potential usefulness of therapeutic protocols based on EBV has been demonstrated recently by Neyts *et al.*[18] who studied xenografts of EBV-associated nasopharyngeal carcinomas in athymic nude mice. Administration of the antiviral agent *Cidofovir* had a pronounced antitumor effect in these tumor-bearing mice, apparently as a result of rapid cell death, through apoptosis, of EBV-transformed epithelial cells.

Primary effusion lymphomas have a more complex association, not with one but with two distinct viruses of the herpes group, namely EBV and human herpesvirus-8 (HHV-8), the Kaposi's sarcoma associated agent. This has recently been described by Horenstein *et al.*[19] The various patterns of EBV latency expression and the interaction with HHV-8 may contribute to a better understanding of the pathobiology of this form of lymphoproliferative disease.

The association between HHV-8 infection and multiple myeloma was initially reported by Said *et al.*[20] in 1997 and confirmed by Broussais *et al.*[21] However,

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Cathomas *et al.*,[22] in Switzerland, had difficulties in confirming the PCR results and stressed the absence of anti-HHV-8 antibodies in 17/18 multiple myeloma patients. In sharp contrast, anti-HHV8 antibodies were readily identified in the majority of a group of patients with Kaposi's sarcoma.

The fact, initially reported in 1994, that a large majority of cases of Kaposi's sarcoma are associated with a virus of the herpes group, HHV-8, seems generally accepted. This observation may have interesting therapeutic implications as indicated recently by Low *et al.*,[23] working in Fleckenstein's laboratory in Erlangen. Low observed a transient disappearance of HHV-8 DNA in the PBMC of a patient with disseminated Kaposi's sarcoma. The positive PCR results in PBMC were interpreted as reflecting *viremia*, although viremia classically means the presence of free virus particles in blood plasma.

Considerable progress has been made in the understanding of virus-host cell interactions. Thirty years ago, viruses were subclassified into cytolytic and non-cytolytic. The Epstein-Barr virus was regarded as cytolytic because it was, most of the time, observed by electron microscopy in cells apparently in a state of degeneration, with pyknotic nuclei. Important advances have been made in the understanding of apoptosis, which is modulated by many positive and negative controls. For example, the overexpression of the anti-apoptotic BCL-2 protein contributes to some forms of cancer, while the loss of p53 function reduces sensitivity of cells to the apoptosis inducing activity of genotoxic drugs or irradiation. These fundamental aspects of cell growth control are currently studied in many laboratories, including the St. Mary's Branch of the Ludwig Institute in London.[24] That these aspects of cell growth control are somehow related to viral infection, and in particular to EBV infection is clearly indicated by recent observations made in Erlangen by Fleckstein *et al.*,[25] where the main research emphasis is placed on the anti-apoptotic strategies of lymphotropic viruses, *evasion of cytotoxic T-cell effects* being part of these strategies.

Papova viruses

Human papillomaviruses (HPV), mainly types 16 and 18, are believed to be responsible for the development of invasive cancer of the uterine cervix. It is still not clear, however, whether HPV is a passenger, a driver, or both, as recently discussed by Leopold Koss[26] in New York. In an early PCR study, 46% of a group of healthy female university students were shown to carry the virus. It is unlikely that such a number of young women are candidates for cervical cancer; most likely HPV is only a *passenger* in most cases. For many oncologists there seems to be little doubt that HPV plays some role in human cancer. However, what transforms a passenger virus into a driver is still an open question. One interesting line of research is related to possible interactions between a protein product of the open reading frames E6 and E7 with protein products of cancer inhibitory genes Rb and p53. Type 16 HPV E7 is a viral oncoprotein, which plays a major role in cervical neoplasia according to Wang-Johanning *et al.*[27] who prepared antibodies against this oncoprotein with possible therapeutic applications.

The identification of HPV subtypes seems to contribute little to the clinical management of patients, as already indicated by Koss and recently confirmed by Herrington *et al.*[28]

Pathogenicity of human papillomavirus is not restricted to the uterine cervix. As well documented by Steinberg *et al.*,[29] in 1996, HPVs cause benign tumors in the respiratory tract, and probably play a role in the etiology of a subset of head and neck cancers. Here again, HPV-16 and 18 are associated with a higher probability of malignant conversion (*high risk* viruses). Clues to the mechanism of action of E7 are discussed by these authors in terms of possible interaction with several cell-cycle regulator proteins which may further contribute to abnormal cell cycle progression. However, approximately 1/3 of all women with cervical cancer have never been infected with HPV. Therefore, HPV could possibly be a co-factor in some cases. In HPV positive patients, however, one wonders whether possible anti-viral therapy might be considered in view of the fact, reported by Neyts *et al.*,[18] that the anti-DNA virus *Cidofovir* produces complete regression of Shope papilloma virus-induced lesions in rabbits.

General considerations on studies related to the hypothetical viral etiology of some human cancers

The two examples just considered - EBV and HPV - clearly indicate that contemporary viral oncology research is primarily based on the identification of viral markers such as proteins or nucleic acids.

However, the specificity of viral markers depends on the success of virus isolation and purification. Without fully demonstrated success in virus isolation and purification, identification of *viral markers* is extremely hazardous and can lead to severe misinterpretation of clinical data. A dramatic illustration of this is to be found in current HIV research. In this case, the virus (HIV) has never been properly isolated, since sedimentation in sucrose gradient at the density of 1.16 g/mL was erroneously considered to yield *pure virus*, systematically ignoring that material sedimenting at that density contains large amounts of cell debris and microvesicles.[30,31] Therefore, proteins and nucleic acids found in such *1.16 bands* are very likely to be of cellular origin and cannot be used as viral markers. Such a faulty methodology has had extremely serious consequences, i.e. the world-wide use of HIV-antibody tests, Elisa and Western Blot, which dangerously lack specificity, as demonstrated in 1993 by Papadopulos *et al.*,[32] in Australia.

Admitting, however, that some viral markers are

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specific, their presence within tumor cells will probably never show more than an association. Etiological relationships are unlikely to be demonstrated by the presence of markers, even if these markers are related to the viral genome. One has difficulties in following Levin and Levine[33] when they state that the identification of the viral genome in tumor cells is *the strongest evidence for its activity as an oncogenic agent*. This is reminiscent of an old problem when electron microscopy was only showing association with viruses, but never their etiological significance.

In microbiology, most viral diseases are highly contagious. If some forms of cancer had viral etiology, how is it that we don't see more cancer *clusters*? Clusters have been occasionally observed, but their number is very small and is certainly not compatible with the concept of primary infections. We know that EBV is a ubiquitous virus. And, as T. Osato[10] points out, *ubiquity and oncogenicity are seemingly incompatible*. But we are not aware of the ubiquity of HHV-8, and we don't see any evidence for clusters of HHV-8 associated malignancies.

An area in which progresses have been highly significant is unquestionably that of apoptosis. Thirty years ago, viruses were regarded as either cytolytic or non-cytolytic. This property was considered as an intrinsic characteristic of the virus itself. Today, factors controlling cell cycling are much better understood, and the cell cycle appears as a fragile balance between apoptotic cell death and cell immortalization. Suppression of apoptosis may contribute to cancer. As studied at the Ludwig Institute in London,[24] it appears, for example, that over-expression of the anti-apoptotic BCL-2 protein is a key event in follicular lymphoma. Factors interfering with the progression through the cell cycle are many; some are endogenous, some are exogenous; some are chemical in nature, others are physical; some could probably be added by the activation of latent viruses, such as EBV. All experiments supporting this view are, however, *in vitro* experiments, and it will take considerable clinical skill to demonstrate that these *in vitro* observations are of any significance in the sudden development of tumors in latently EBV infected individuals.

If viral markers show only *association*, without implying etiology, this does not mean that the presence of such markers within cancer cells is not of possible therapeutic usefulness. *Targeting* is an interesting approach to chemotherapy, or to CTLs lymphocytes. A significant example for this can be found in the paper by Roskrow *et al*.[17] on EBV-specific cytotoxic T lymphocytes for the possible treatment of patients with EBV-positive relapsed Hodgkin's disease.

But what about antiviral therapy? Could it possibly be that its eventual success would produce the evidence for the oncogenicity of some viruses which we are so eagerly trying to establish? When we learned that *Cidofovir* produces complete regression of Shope papilloma virus-induced lesions in rabbits and that its nephrotoxicity can be kept under control in humans,[18] it became a most attractive approach to therapy, as well as to understanding of the still hypothetical oncogenicity of the *associated* virus. Can the effects of Foscarnet on Kaposi's sarcoma and HHV-8 associated hemophagocytic syndrome[23] possibly be placed in the same perspective?

For DNA viruses associated malignancies, we have effective antiviral agents of manageable toxicity at hand. This is not the case for RNA virus associated diseases, and in particular for syndromes such as AIDS, hypothetically associated[6,32] with infection by the HIV retrovirus. In these cases, the currently used combined antivirals are unacceptably toxic, making the so-called *therapy* worse than the disease itself! Moreover, the effects of anti-retroviral therapy are currently measured by *quantitative* PCR technology. Unfortunately, Karry Mullis PCR technology is not reliable to measure what has been erroneously labeled *viral load* in AIDS patients.[34,35]

As a concluding remark, I wish to say that the cases for both herpes and papova viruses are worth considerable attention. This is in sharp contrast with *retroviruses*, which, to the best of my knowledge, have never been satisfactorily demonstrated to be associated with any human disease.

Etienne de Harven, MD
Professor Emeritus of Pathology,
University of Toronto, Ontario, Canada

Correspondence:
"Le Mas Pitou", 2879 Route de Grasse,
06530 Saint Cézaire-sur-Siagne, France.
Tel & Fax: international +33-4-93602839
E-mail: pitou.deharven@wanadoo.fr

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Haematologica 1999; 84:390-396 original paper

In vitro growth and quantification of early ($CD33^-/CD38^-$) myeloid progenitor cells: *stem cell factor* requirement and effects of previous chemotherapy

DARIO FERRERO, CRISTINA CHERASCO, BARBARA ORTOLANO, FULVIA GIARETTA, BENEDETTO BRUNO
Divisione di Ematologia dell'Università di Torino, Azienda Ospedaliera "San Giovanni Battista", Turin, Italy

ABSTRACT

Background and Objective. **All culture systems exploring the early (pre-CFU) hematopoietic compartment are generally complex, time-consuming and unsuitable for routine application. The aim of our study was to develop a stroma-free culture system to quantify early bone marrow (BM) myeloid progenitor cells.**

Design and Methods. **Low density, progenitor cell enriched BM cells underwent a double cytotoxic treatment with CD38 and CD33 monoclonal antibodies + rabbit complement, which depleted 99% of CFU-GM and BFU-E. Then they were cultured, both in agar and in limiting-dilution liquid culture, in the presence of 5637 cell line supernatant (containing GM-CSF, G-CSF and interleukin 1), stem cell factor (SCF) and interleukin 3 (IL3).**

Results. **The largest number (median 14.9 on 1×10^5 cells) and size (>50,000 cells) of myelomonocytic cell clones from $CD33^-/CD38^-$ progenitors was reached after 3-4 weeks of liquid culture. SCF, but not IL3, was essential for that growth. The frequency of $CD33^-/CD38^-$ progenitors grown in liquid culture was approximately three times greater than the LTC-IC frequency in the same cell suspension. An average 93% of $CD33^-/CD38^-$ progenitors displayed HLA-DR antigens and 43% generated secondary CFU-GM. In the BM of 9/10 patients, previously exposed to chemotherapy, $CD33^-/CD38^-$ progenitor frequency was quite low (median 0.9 on 1×10^5 cells), in spite of normal cellularity and morphology and sustained disease remission.**

Interpretation and Conclusions. **$CD33^-/CD38^-$ progenitors can be grown and quantified in a stroma-free culture system in a relatively short time. The test can reveal long-lasting, subclinical BM damage induced by chemotherapy and could also be valuable for estimating the amount of early myeloid progenitors for transplantation purposes.**
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Key words: CD38 negative progenitors, complement cytotoxicity, stem cell factor, chemotherapy

Correspondence: Dario Ferrero, M.D., Divisione di Ematologia dell' Università di Torino, Azienda Ospedaliera "San Giovanni Battista", via Genova 3, 10126 Turin, Italy.
Phone: international +39-011-6336728/6335329 – Fax: international +39-011-6963737.

The progressive development, during the past two decades, of *in vitro* culture techniques has allowed routine evaluation of the growth of committed hematopoietic progenitors. These cells can be quantified in bone marrow (BM) and peripheral blood for transplantation and other clinical purposes.[1,2]

Conversely, *in vitro* culture and quantification of early hematopoietic progenitor/stem cells is still a difficult task. Some techniques for this purpose use pre-established BM stromal layers, without exogenous growth factors.[3-5] In particular, in the past few years, a culture system has been developed that supports the growth, in microwells with irradiated BM stroma, of so called *long-term culture-initiating cells* (LTC-IC). The method allows quantification of these very primitive cells that share many of the features of hematopoietic stem cells.[3,6] However, LTC-IC assay is a complex procedure, requiring a pre-established irradiated BM layer and a long culture time (at least five weeks), this precluding its extensive use in a clinical setting.

Stroma-free cultures require the use of combinations of growth factor[6,7] and of selective procedures to suppress the overgrowth of the more abundant committed progenitors. Depletion of committed progenitors can be achieved by the use of selective culture medium,[7,8] prolonged culture time,[9] cytotoxic drugs[10,11] or monoclonal antibodies against differentiation or proliferation antigens.[7,11-14] Indeed, the earliest hematopoietic cells lack lineage-specific as well as CD38 and CD71 antigens, which are expressed by committed progenitor cells.[13-15] In particular, CD38 antigen is present on almost all hematopoietic colony-forming cells.[13] Therefore, CD38 negative progenitors represent a small fraction of very immature hematopoietic cells that lack lineage-specific antigens, and include LTC-IC.[16] Here we describe some growth requirements of $CD33^-/CD38^-$ myeloid progenitors and describe a relatively short, stroma-free, culture method for their quantification in human adult BM. We also report the observation, using this method, of a great reduction in $CD33^-/CD38^-$ progenitor cells in the BM of patients previously treated with cytotoxic drugs, in spite of normal blood counts and BM cellularity.

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Design and Methods

Cells

BM was aspirated from volunteer donors and from patients undergoing diagnostic procedures after informed consent had been obtained. Low density (< 1,077 g/L) cells, obtained by Ficoll/metrizoate (Lymphoprep, Nycomed) density gradient separation, underwent a second Lymphoprep separation after phagocytosis of opsonized, heat-inactivated yeast, in order to remove mature myelo-monocytic cells.[17] The separation procedure resulted in a 6-10 fold enrichment in myeloid and erythroid progenitors.

Complement-dependent cytotoxicity

One million low density non-phagocyting cells (LD/phag⁻), suspended in 0.25 mL of Iscove's modified Dulbecco's Medium (IMDM) with 10% fetal bovine serum (FBS), were incubated at 4°C in the presence of 10 µL of CD33 and CD38 monoclonal antibodies (MoAb). CD33 MoAb was purchased from Coulter. CD38 was initially purchased from Technogenetics, then, in further experiments, we employed the MoAb produced by the IB4 clone, kindly provided by Prof F. Malavasi (Istituto di Genetica Medica, Università di Ancona). In some experiments, 10 µL of anti-HLA-DR MoAb (Becton-Dickinson) were also included in the cytotoxicity test. After 30' of incubation with MoAb, 0.27 mL of rabbit complement (GIBCO), previously adsorbed on human AB group erythrocytes and diluted 1:3 with IMDM, were added (final dilution 1:6) and the incubation was continued at 37°C for 60'.[18] Cells were then washed once and re-exposed to MoAbs and complement. Finally, cells were washed once with IMDM and resuspended in 1 mL of IMDM+10% FBS. Control cells underwent the same incubations without either antibodies or complement or both. The efficacy of cytotoxic treatment was checked by direct immunofluorescence using a phycoerythrin-labeled CD38 MoAb (Becton-Dickinson) and FACS analysis.

Colony assays

Forty microliters of control or 100 µL of MoAb-treated cells were seeded in each of two 35 mm Petri dishes containing 1 mL of IMDM + 20% FBS + 0.3% agar (DIFCO) + 10% supernatant of 5637 cell line (as a source of GM-CSF, G-CSF and interleukin 1)[19,20] ±30 ng/mL of human recombinant *stem cell factor* (SCF) (purchased from Amgen). In some experiments, 15 ng/mL of human recombinant interleukin-3 (IL3) (purchased from Sandoz) and 2 U/mL of human recombinant erythropoietin (Eprex®, from Cilag) were also added to allow BFU-E growth. The amount of control cell suspension seeded in each dish corresponded to 4×10^4 cells, since no significant cell loss occurred during incubations or centrifugations. One hundred microliters of MoAb-treated cell suspension corresponded to 1×10^5 LD/Phag⁻ cells before cytotoxicity; no correction was made for the actual number of cells which survived cytotoxicity, in order to avoid an enrichment in CD33⁻/CD38⁻ cells. Colonies were scored after 14 and 28 days of incubation at 37°C in 5% CO_2 atmosphere.

Liquid culture

Each of four groups of twenty-four flat bottomed microwells of a 96 microwell plate (Falcon, Becton-Dickinson) was seeded with a different amount of MoAb-treated cell suspension: 10 µL, 5 µL, 2 µL and 1 µL/microwell. These volumes corresponded, respectively, to 10,000, 5,000, 2,000 and 1,000 LD/phag⁻ cells before cytotoxicity. Each microwell was filled with 100 µL of IMDM containing 20% FBS, 10% 5637 cell line supernatant, 30 ng/mL SCF and 15 ng/mL IL3. In some experiments, either SCF or IL3 or both were omitted from the culture medium. After 7 days of culture, 50 µL of medium were removed from each well and replaced by an equal volume of fresh medium and growth factors, then, at the 14th day, 50 µL of fresh medium were added. From the 14th to the 35th day of culture, microwells were scored weekly for the presence of evident cell growth (more than 100 alive cells). The cloning efficiency, calculated on the basis of the proportion of growth-negative wells by Poisson statistics and weighted mean method,[21] corresponded to the reciprocal of the cell concentration that determined absence of growth in 37% of wells (Figure 1). Cloning efficiency was expressed as number of clones/1×10^5 starting (pre-cytotoxicity) cells.

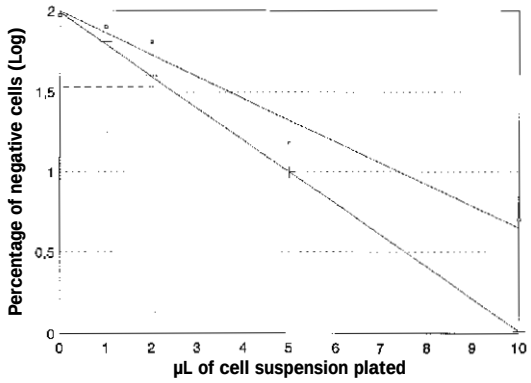

Figure 1. Limiting dilution assay of CD33⁻/CD38⁻ progenitor cells.
One million LD/phag⁻ cells underwent cytotoxic treatment with CD33 and CD38 MoAb + complement, as described in the *Design and Methods* section; they were then re-suspended in 1 mL of culture medium. Different volumes of such a cell suspension (each µL corresponding to 1000 cells before cytotoxicity) were seeded into groups of 24 wells and cultured as described in the *Design and Methods* section. The percentages of wells with and without cell growth were scored after 3-4 weeks. Values on the Y axis represent the Log_{10} of the percentage of negative wells and the two curves refer to different BM samples. The cloning efficiency, calculated by Poisson statistics and the weighted mean method,[21] corresponds to the reciprocal of the cell concentration that determined 37% of negative wells, (dashed line).

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In some experiments, cells from wells likely to contain a single clone (rows with less than 30% growth-positive wells)[21] were harvested for counting, morphologic examination and CFU-GM assay. Adherent macrophages were detached after exposure to a 5% trypsin/EDTA solution (GIBCO) for 15' at 37°C, then pooled with suspension cells from each well. Slides, obtained by spinning in a Shandon cytocentrifuge, were stained with May-Grünwald-Giemsa stain.

In order to test CFU-GM generation from CD33⁻/CD38⁻ progenitors, clones were harvested after 2-3 weeks of liquid culture and each was replated in a well of a 24 well plate, containing 0.4 mL IMDM + 20% FBS + 10% 5637 cell line supernatant + 0.9% methylcellulose (Dow, 4000 cps). Secondary colonies were scored after 14 days.

LTC-IC assay

The same volumes of CD33⁻/CD38⁻ cell suspension used for the above described limiting-dilution cultures were seeded into 96 flat bottomed microwells, each containing BM stroma, subcultured from previously established irradiated layers,[3] and 100 µL of IMDM with 12% FBS, 12% horse serum, 10^{-6} M hydrocortisone and 10^{-4} M 2-mercaptoethanol.[3] After 5 weeks, all suspension and adherent cells from each well were harvested, as above described, and cultured for CFU-GM assay (see above). LTC-IC frequency was calculated from the proportion of CFU-GM-negative wells.[21]

Statistics

The Wilcoxon matched-pairs signed-ranks test was used to analyze the difference in colony growth in the presence/absence of SCF. The differences in the concentration of total CFU-GM and CD33⁻/CD38⁻ progenitors in the BM of untreated and chemotherapy-exposed subjects was evaluated by the Wilcoxon-Mann-Whitney test.

Results

Cytotoxicity test and growth in semi-solid medium of CD33⁻/CD38⁻ progenitor cells

The treatment with CD33 + CD38 MoAb plus complement almost totally prevented CFU-GM and BFU-E growth in agar medium containing 5637 SN, IL3 and EPO. Indeed, the median number of residual myelomonocytic colonies was 0.9% (range 0-5%) (Table 1) of values detected in control cultures. The growth of erythroid colonies was also strongly suppressed after cytotoxic treatments in three experiments, with values in control and antibody-treated cultures of 120, 36, 172 and 0.5, 0, 2.5, respectively (average residual growth after cytotoxicity: 1%). Mixed (CFU-GEMM derived) colonies, occasionally observed in control cultures, were never detected after cytotoxic treatment. A 99% reduction of total CD38⁺ cells was also detected by direct immunofluorescence (data not shown). Conversely, control treatments with either MoAbs or complement alone did not significantly reduce colony formation (98% average CFU-GM growth, compared to untreated cells).

In 11 experiments SCF was included in the culture medium containing 5637 SN±IL3: some myeloid colonies (median value 11 on 1×10^5 LD/phag⁻ cells, range 3.5–67.5) constantly appeared from cells treated with MoAbs and complement (CD33⁻/CD38⁻ cells), reaching a median of 5% (range 1-19%) of control CFU-GM values. The increment in colony growth from CD33⁻/CD38⁻ cells induced by SCF was highly significant (p: 0.0044) and reached a median value of 910% (Table 1); conversely SCF increased by a median of only 32% (p: 0.013) the number of colonies arising from total LD/phag⁻ cells (control cultures) (Table 1). Colonies arising from CD33⁻/CD38⁻ cells reached their maximum size after 3 weeks, surviving until the 4th week; their number was unaffected by the presence or absence of IL3 (data not shown).

Limiting-dilution liquid cultures

Cell growth became evident, in some microwells, after the second week of culture. The frequency of CD33⁻/CD38⁻ progenitor cells giving rise to clones in liquid culture was calculated between the 21st and 28th day of culture, when the greater proportion of growth-positive wells was reached (Figure 2). That frequency was found to range, in normal BM samples, between 8.9 and 84.3 on 1×10^5 LD/phag⁻ cells (median value 14.9) (Table 2). In 14/15 tests the frequency of CD33⁻/CD38⁻ progenitor cells appeared to be somewhat higher (median +49%) in limiting-dilution liquid culture than in agar medium colony-assay, in the presence of the same growth factor combination (compare Table 2: *controls* to Table 1: *CD33⁻/CD38⁻ progenitors in the presence of SCF*). The largest clone size was usually reached after 4 weeks of culture, most clones including several thousands of cells (5,000-50,000) of the granulo-monocytic lineage. Only a few clones contained relatively small numbers of cells (100-500),

Table 1. Effects of SCF on the growth of total CFU-GM and CD33⁻/CD38⁻ progenitor cells in agar medium.

		Colony values*			
Progenitors	*Scf*	*Mean*	*Median*	*Range*	*Statistics°*
Total CFU-GM	–	115	104	37-240	
Total CFU-GM	+	147	136	48–326	p 0.013
CD33⁻/Cd38⁻	–	3	1	0-11	
CD33⁻/Cd38⁻	+	22	11	3-67	p 0.004

**Total CFU-GM values expressed as colonies/$4x10^4$ untreated (controls) LD/Phag⁻ cells; CD33⁻/CD38⁻ progenitors expressed as colonies/$1x10^5$ LD/Phag⁻ cells exposed to CD33 and CD38 MoAb + complement, as described in the Design and Methods section.*

°Statistical analysis, by Wilcoxon matched-pairs signed-ranks test, of SCF-induced differences in colony growth.

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Table 2. Frequency of total CFU-GM and CD33⁻/CD38⁻ progenitors among BM cells of controls and chemotherapy-treated patients.

Controls			*Chemotherapy-treated*			
Case #	*CFU-GM/ 40,000 cells^*	*CD33⁻/CD38⁻ 100,000 cells^*	*Case #*	*Diagnosis°*	*CFU-GM/ 40,000 cells^*	*CD33⁻/CD38⁻ 100,000 cells^*
1	200	49.4	1	NHL	99	7.2
2	290	58.9	2	MM	99	0.8
3	284	8.9	3	MM	138	1.2
4	240	13.3	4	MM	105	0.3
5	142	13.9	5	HD	71	0.3
6	71	26.7	6	AML	95	0.6
7	62	25.3	7	MM	73	6.2
8	326	24.5	8	ALL	121	6.1
9	185	13.4	9	AML	23	0.3
10	81	9.9	10	NHL	172	71.0
11	178	84.3				
12	121	15.9				
13	120	10.8				
14	103	10.2				
Median	160	14.9	Median		99	1.0

**Normal donors or untreated patients without disease involving the myeloid lineage; ^Low density cells depleted of phagocyting cells (LD/Phag⁻). °NHL: non-Hodgkin's disease; MM: multiple myeloma; AML: acute myeloid leukemia; ALL: acute lymphoid leukemia.*

usually macrophages. Erythroid cells were observed occasionally in some microwells during the 2nd week of culture, when EPO was present in the culture medium, but later disappeared.

Growth factor requirement

The omission of SCF from the culture medium resulted in more than one log decrease in the cloning efficiency of CD33⁻/CD38⁻ cells in liquid culture, whereas only a minimal, not significant growth reduction was determined by the absence of IL3 (Figure 3). SCF alone did not induce any clone growth (data not shown). Further characterization of CD33⁻/CD38⁻ myeloid progenitors was performed by limiting-dilution liquid culture in medium containing 5637 SN + SCF + IL3.

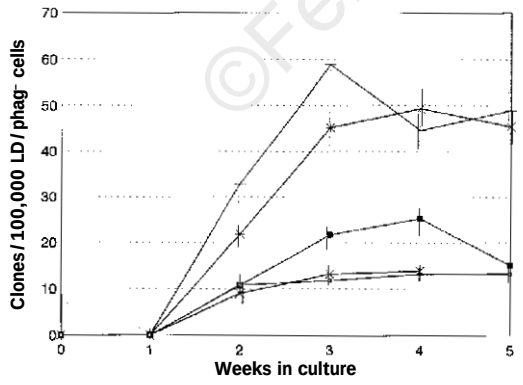

Figure 2. Time-course appearance of clones from CD33⁻/CD38⁻ progenitor cells in liquid culture.
LD/phag- BM cells underwent cytotoxic treatment with CD33 and CD38 MoAb + complement, and culture at limiting-dilution in the presence of 5637 SN, SCF and IL3. Clone numbers were scored at weekly intervals and their frequencies were calculated as described in the *Design and Methods* section. Curves refer to different BM samples.

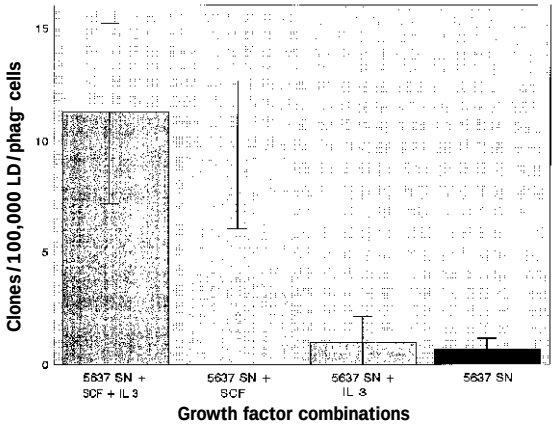

Figure 3. Growth of CD33⁻/CD38⁻ progenitor cells in liquid medium with different growth factor combinations.
LD/phag⁻ BM cells underwent cytotoxic treatment with CD33 and CD38 MoAb + complement. They were then cultured at limiting-dilution in liquid medium containing 5637 SN with/without SCF and/or IL 3. Clones were scored after 4 weeks of culture and their frequencies were calculated as described in the *Design and Methods* section. Bars represent the mean (±SD) value of three experiments with different BM samples.

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Expression of HLA-DR molecules

The expression of HLA-DR molecules by CD33⁻/CD38⁻ progenitors was checked in four experiments by a parallel cytotoxicity test that included anti-HLA-DR together with CD33 and CD38 MoAbs. Anti-HLA-DR MoAb reduced the frequency of CD33⁻/CD38⁻ clonogenic cells by more than one log (average recovery 7.5±1.3%).

CFU-GM generation from CD33⁻/CD38⁻ progenitors

Seventy-six clones, from 3 experiments, were re-plated in methylcellulose medium for CFU-GM assay. Thirty-three clones (43%) showed the presence of CFU-GM, as demonstrated by the growth of variable numbers of colonies (1-98, median value: 5). Most of these colonies survived in semisolid culture until the third week; they were, however, of smaller size (50–500 cells) than primary clones. In one experiment secondary colonies were harvested, pooled and re-plated but no tertiary colonies were detected (data not shown).

LTC-IC

In three experiments a direct comparison was made between the frequency of CD33⁻/CD38⁻ progenitor cells growing in stroma-free liquid culture and LTC-IC. As shown in Table 3, a fair correlation was found between the two values, LTC-IC corresponding to 29±2% of CD33⁻/CD38⁻ progenitor cells.

CD33⁻/CD38⁻ progenitors in the BM of chemotherapy-treated patients

The frequency of CD33⁻/CD38⁻ progenitors and of total CFU-GM was evaluated in BM samples from 10 patients affected by different hematologic malignancies (Table 2). All patients had undergone different cytotoxic treatments 2-60 months before the test and were in complete remission, with normal blood counts and BM morphology. The proportion of LD/phag⁻ cells was also similar (10-15%) in chemotherapy-treated and in control BM samples (data not shown). However, all but one patient displayed a quite low frequency of CD33⁻/CD38⁻ progenitor cells (median value 1 on 1×10^5 LD/phag⁻ cells, range 0.3-71): the difference from values observed with normal BM samples was highly significant (p=0.0009). Conversely, total CFU-GM frequency was only moderately reduced in patients' (median value 99 on 4×10^4 LD/phag⁻ cells, range 23-172) compared to control BM samples (median 181 on 4×10^4 LD/phag⁻ cells, range 62-326) (p=0.04). The size of clones from both total CFU-GM and CD33⁻/CD38⁻ progenitors was not different in control and patients' cultures.

Table 3. Comparison of CD33⁻/CD38⁻ progenitor growth in stroma-free liquid culture and in LTC-IC assay.

Experiment	Clones* in stroma-free culture	LTC-IC°
1	13.2±2.5	4.6±1.2
2	58.9±10.7	17.9±2.8
3	49.4±8.8	11.4±2.1

*CD33⁻/CD38⁻ cells, obtained by complement-dependent cytotoxicity from three normal BM samples, were cultured at limiting-dilution in stroma-free liquid culture, in the presence of 5637 SN+ IL3 + SCF, and in stroma–containing microwells for LTC-IC assay. *values refer to the number of clones on 1×10^5 pre-cytotoxicity LD/Phag⁻ cells, evaluated as described in the Design and Methods section; °values refer to the number of LTC-IC on 1×10^5 pre-cytotoxicity LD/Phag⁻ cells, evaluated as described in the Design and Methods section.*

Discussion

The aim of our study was to establish a stroma-free culture system capable of quantifying, in 3-4 weeks, early myeloid progenitor cells (pre-CFU-GM) in normal BM and in chemotherapy-treated patients. These progenitors can be identified by immunofluorescence as CD33⁻/CD38⁻ cells.[13,15] However, only 10-25% of cells with such a phenotype are clonogenic *in vitro* and identifiable as LTC-IC or *blast* colony-forming cells;[6,13] moreover, they cannot be accurately quantified by immunofluorescence in BM samples because of their low frequency (approximately 1% of total CD34⁺ cells, 0.01% of the whole cellularity in normal samples),[13] which is at the lower limit of detection by FACS analysis. Conversely, a clonogenic assay can provide more precise information, particularly in cases with a lower than normal frequency.

Depletion of more differentiated CFU-GM was achieved by MoAbs (in complement-dependent cytotoxicity) that, in our experience, gives more reproducible results than cytotoxic drugs such as cyclophosphamide metabolites. CD38 antigen was reported to be expressed by all lineage-committed hematopoietic progenitors, but we also added CD33 MoAb in the cytotoxicity assay to ensure the maximal killing of mature progenitor cells. Indeed, the 99% reduction of myeloid and erythroid colonies in standard culture conditions was indicative of a virtually complete depletion of CD33⁺ and CD38⁺ cells. This also avoided the need for morphologic analysis and/or recloning of all the clones grown in liquid culture in order to prove their origin from early progenitor cells, as required by some *blast cell* colony assays.[8-10]

The optimal growth of CD33⁻/CD38⁻ progenitor cells was obtained in liquid culture, in the limiting-dilution assay. The long growth time and the large size of clones suggested that they originated from early progenitor cells. This was also confirmed by the presence, in 43% of clones, of CFU-GM capable of giving rise to secondary colonies, in agreement with results obtained by another group with CD34⁺/CD38⁻ cells.[6,13]

SCF was absolutely essential for CD33⁻/CD38⁻ cell growth, both in agar and in liquid cultures, whereas IL3 did not play a significant role. Therefore, a combination of growth factors, and particularly the presence of

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SCF and 5637 SN (containing GM-CSF, G-CSF and IL 1),[19,20] are required for an optimal growth of CD33⁻/CD38⁻ progenitors. SCF was reported to improve CFU-GM and BFU-E growth greatly in the presence of sub-optimal concentrations of CSF and EPO.[22] However, SCF activity on CFU-GM is less evident in the presence of optimal CSF combinations, as observed in our experiments with control cells stimulated by 5637 SN (Table 1). Conversely, SCF was absolutely essential for CD33⁻/CD38⁻ progenitor cells, in spite of the optimal 5637 SN concentration. In a previous report, CD38- progenitor cells were efficiently stimulated to form blast colonies by a growth factor mixture that did not contain SCF but included IL6, IL3, GM-CSF, G-CSF and EPO.[13] Thus, IL6 may substitute SCF in stimulating CD38⁻ progenitors, since it was described to synergize with IL3 in driving quiescent early hematopoietic progenitor/stem cells into proliferative activity.[8] More recent results have, however, reiterated the necessity of SCF, together with Flt3- ligand, IL-3, IL-6 and G-CSF for optimal CD34⁺/CD38⁻ cell growth and LTC-IC expansion *in vitro*.[23,24]

More than 90% of CD33⁻/CD38⁻ progenitor cells were found to express HLA-DR antigens. Moreover no erythroid cells could be generated in long-term culture. Thus CD33⁻/CD38⁻ progenitor cells probably represent a more differentiated cell population than LTC-IC, that lack or minimally express HLA-DR antigens.[3,25] Indeed, it was reported that LTC-IC represent only 10-20% of CD34⁺/CD38⁻ BM cells.[6] However, we found an almost constant ratio of LTC-IC/CD33⁻/CD38⁻ progenitor cells in three of our experiments (Table 2), in spite of a wide variation in their absolute numbers. It is, therefore, possible that a minority of progenitors that formed clones in our culture system actually represented LTC-IC.

The frequency of CD33⁻/CD38⁻ progenitors among LD/phag⁻ BM cells was found to be greatly reduced in 9/10 patients who had received cytotoxic chemotherapy, in spite of normal blood counts and BM cellularity. Since the proportion of LD/phag⁻ cells and total cellularity were comparable to those observed in normal BM samples, a true depletion of CD33⁻/CD38⁻ progenitors occurred in the BM of these patients. The frequency of total CFU-GM too was somewhat reduced in BM of most chemotherapy-treated patients, a finding which is in agreement with results from another group.[26] The differences from control values were not, however, so evident as those observed with earlier progenitors. It is possible that long-term damage to the hemopoietic system caused by cytotoxic drugs may become evident as a reduction of earliest progenitor/ stem cells before a clear depletion of CFU-GM and BM cellularity occurs. Indeed, the only patient with normal values of both total CFU-GM and CD33⁻/CD38⁻ progenitors had been off therapy for four years, after receiving a short-term course of chemotherapy (MACOP-B).[27] This chemotherapy is unlikely to be very toxic for early hematopoietic stem cells because of the use of a single alkylating agent (cyclophosphamide) to a low cumulative dosage. However, the small number of patients and the heterogeneity of their diseases do not allow definitive conclusions to be drawn.

Nevertheless, the quantification of CD33⁻/CD38⁻ progenitors could represent a useful test for ascertaining sub-clinical long-term BM toxicity of anti-neoplastic drugs. Indeed, we have recently confirmed a long-lasting depletion of both CD33⁻/CD38⁻ progenitors and LTC-IC in most patients successfully autotransplanted with BM or peripheral blood progenitor cells (manuscript in preparation).

Moreover, compared to those of other methods exploring the early hematopoietic compartment, the simpler procedure and shorter incubation time of our assay make it valuable for transplantation purposes too, in estimating the amount of immature myeloid progenitors in BM harvests and leukapheresis collections. Indeed, the amount of CD38⁻ progenitor cells autotransplanted has recently been found to predict early myeloid regeneration more accurately[28] than total CD34⁺ cells.[29,30]

Contributions and Acknowledgments

DF was the main investigator: he designed the study and wrote the paper; BO and BB performed most of the cytotoxicity and cell culture experiments; CC performed LTC-IC assays and helped in cell separation procedures; FG did immunofluorescence tests for CD38 MoAb titration and cytotoxicity evaluation. The first and last authors had the main roles in performing this study, the order of the other authors was decided on the basis of the amounts of contribution they gave to the experiments.

The authors wish to thank Dr. Chiara Argentino (Divisione di Ematologia, Università di Torino) and Dr. Gabriele Gallone (Azienda Ospedaliera "Maria Vittoria", Torino) for their help with the statistical analysis.

Funding

This study was supported by grants from the "Ministero della Pubblica Istruzione" (M.P.I. 40%) and "Associazione Italiana per la Ricerca sul Cancro" (A.I.R.C), Italy.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received November 19, 1998; accepted March 3, 1999.

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Haematologica 1999; 84:397-404 original paper

Clinical and molecular follow-up by amplification of the CDR-III IgH region in multiple myeloma patients after autologous transplantation of hematopoietic CD34+ stem cells

GIOVANNI MARTINELLI, CAROLINA TERRAGNA, ROBERTO M. LEMOLI, MICHELE CAVO, MONICA BENNI, MARIA ROSA MOTTA, MARILINA AMABILE, EMANUELA OTTAVIANI, NICOLETTA TESTONI, ANTONIO DE VIVO, SANTE TURA

Institute of Hematology and Medical Oncology "Seràgnoli", University of Bologna, Italy

ABSTRACT

Background and Objective. **Autologous blood stem cell transplantation (ABSCT) using chemotherapy-induced mobilization of peripheral blood stem cells (PBSC) is being increasingly used in the treatment of multiple myeloma (MM). We report the clinical and molecular follow-up of 10 MM patients who underwent autologous stem cell transplantation with peripheral blood selected CD34+ cells, as support therapy following a myeloablative conditioning regimen.**

Design and Methods. **The CDR-III coding region of the IgH gene was studied by a) consensus PCR applied to 8 MM patients, or b) by direct sequencing of PCR product generated by family-specific primers in the remaining two patients (who became immunofixation analysis (IF) negative). In this case, two patient-specific primers were generated, thus obtaining a high PCR assay sensitivity and specificity (ASO PCR).**

Results. **Seven patients are alive: 4 of them have serum M protein assessable by IF, while 1 was not a secretor and 2 converted from serum IF positivity to negativity 6 and 12 months after ABSCT. Three patients died: 1 from disease progression and 2 from infective complications during clinical remission. The molecular analysis during the follow-up showed that the bone marrow samples from the two patients who obtained IF negativity were persistently PCR positive for the presence of rearranged CDR-III region. Moreover, despite the remarkable reduction of myeloma burden, a minimal level of residual myeloma cells was still detectable by molecular analysis.**

Interpretation and Conclusions. **These results confirm that although positive selection of CD34+ cells markedly reduces the contamination of myeloma cells from apheresis products by up to 3 log, and provides a cell suspension capable of restoring normal hematopoiesis after ablative conditioning regimen, it does not abrogate myeloma cell contamination in most of the apheresis products.**
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Key words: minimal residual disease, multiple myeloma, CD34+ cells, ABMT

Correspondence: Giovanni Martinelli, M.D., Institute of Hematology and Medical Oncology "Seràgnoli", Ospedale S. Orsola, via Massarenti 9, 40138 Bologna, Italy. Phone: international +39-051-6364075 – Fax: international +39-051-398973 – E-mail: gmartino@kaiser.alma.unibo.it

Multiple myeloma (MM) is a B-cell-derived neoplastic disease generally associated with the expansion of mature plasma cells, monoclonal Ig production, and multiple osteolytic lesions.[1] Because of the limitations of conventional therapeutic approaches, several regimens involving myeloablative radiochemotherapy followed by the reinfusion of autologous stem cells have been employed.[2-7] The use of autologous stem cells could reduce the morbidity and mortality associated with high-dose therapy-related procedures.[8,9] Thus, many investigators have stressed the option of using blood as an alternative source of autologous hematopoietic stem cells for autografting.[5,9]

Autologous blood stem cell transplantation (ABSCT) using chemotherapy mobilized cells (PBSC) is being increasingly used in the treatment of MM.[9-11] One of the major arguments in favor of ABSCT is the possibility that PBSC harvests may be less contaminated by myeloma cells than bone marrow mononuclear cells (BMMNC).[10,11] However, tumor-related B cells bearing the same idiotypic determinants as neoplastic plasma cells have been identified in the blood of MM patients and they have been shown to belong to the neoplastic stem cell compartment.[12]

Polymerase chain reaction (PCR) has been used for monitoring minimal residual disease (MRD) in MM by amplification of the CDR-III region.[13] Clonal CDR-III region products can be generated by amplification in all instances of MM, thereby providing a specific diagnostic marker for each B cell clone. In fact, the variable regions of immunoglobulin heavy chains (IgH) are encoded by a series of discontinuous germline gene segments, called variable (VH), diversity (D) and joining (JH) regions, which undergo somatic rearrangement at an early stage of B lymphocyte development.[14,15] When a pluripotent hematopoietic stem cell differentiates into the B-cell lineage, the first detectable genetic event is an IgH gene rearrangement in the VH, D and JH regions that consists of two successive somatic recombinations, namely D-JH and VH-D-JH.[16] This region can be considered an identification sequence for members of the same B-cell clone. Rearrangement of the IgH locus in MM presents the

same characteristics and sequence as in normal B-cells.[14,17,18] B-cell lineage hematopoietic malignancies are usually monoclonal and contain one or, more often, two rearranged alleles.[19-21]

It is now well established that CD34+ selected ABSCT leads to stable long term engraftment. In contrast, less information is present in literature regarding the benefit on the response to transplantation, duration of remission and on the level of neoplastic cells in the PB of MM patients after mobilization protocols. Furthermore, tumor cell contamination of patient's bone marrow samples during post-transplant follow-up has been little investigated. We previously reported the analysis of circulating tumor cells by amplification of the CDR-III region in the PBSC of 10 MM patients who underwent ABSCT using CD34+ cells.[9] We now report the clinical outcome and molecular follow-up of the same group of MM patients after ABSCT using CD34+ cells.

Design and Methods

Clinical study

Ten patients with MM diagnosed according to the standard criteria were included in the study (Table 1a). The therapeutic protocol employed was approved by the University hospital ethical committee, and each patient gave written informed consent. Patients were treated with high-dose cyclophosphamide (Cy) (7 g/m^2) as described elsewhere,[9] followed by administration of G-CSF (filgrastim, neupogen, Dompè Biotec, Milan, Italy) at a dose of 5 mg/kg/day s.c. starting on day 2 after chemotherapy and continuing until the completion of PBSC collection. Leukaphereses were performed using a Baxter CS 3000 plus blood cell separator using the modified procedure number 1 program as reported.[9] Circulating hematopoietic CD34+ cells were highly enriched by avidin-biotin immunoabsorption, as previously described,[9] and were cryopreserved prior to use for reconstitution of bone marrow function after myeloablative therapy, immediately following positive selection of CD34+ cells.

CD34+ cells were reinfused on day 0 following the previously reported conditioning regimen.[9] Two patients (#9 and 10) were submitted to a second ABSCT, three months after the first: the conditioning regimen consisted of busulphan (12 mg/kg) (day –5 to –3) and melphalan (120 mg/m^2) (day –2) and was followed by re-infusion of CD34+ cells (day 0). G-CSF at 5 mg/kg/day s.c. was started on day +1 and administered until the granulocyte count had reached >0.5$\times$10^9/L for 3 consecutive days. All patients received a single-donor platelet transfusion if their platelet count was <20$\times$10^9/L and a red blood cell (RBC) transfusion if their hemoglobin level was < 8 g/dL. Patients who achieved a clinical complete remission (CR) or partial remission after transplantation received interferon-α (IFN-α) s.c. (3$\times$10^6 IU/m^2 three times a week), beginning at the time of full hematologic recovery and continuing until there was evidence of disease progression.

Preparation of PB and BM specimens

Preparation of PB and BM specimens for DNA and RNA analyses was performed as described elsewhere.[22] In all patients, the study was performed at diagnosis on bone marrow samples and on aphereses before and after CD34+ selection, and every 3 months during the follow-up. Samples suitable for RNA extraction were collected as reported previously.[23] Serologic CR was defined by the disappearance of the M component at immunofixation analysis, and the resolution of bone marrow plasma cell infiltration to below 3%. Hematopoietic progenitor cell assay, cell phenotype analysis, intracytoplasmic Ig (clg), bromodeoxyuridine (BRDU) staining was performed as reported.[9] Positive selection of CD34+ cells was performed as described elsewhere.[9]

Analysis of minimal residual disease (MRD) by DNA amplification of the CDR-III region of the IgH gene with consensus primers and patients' specific primers

MRD with consensus primers

MRD was analyzed by IgH gene amplification on aphereses as previously described.[9] Briefly, high molecular weight DNA was extracted from bone marrow taken at diagnosis and from leukapheresis collections obtained before and after positive selection of CD34+ cells by proteinase-K digestion followed by phenol chloroform extraction. Amplification was performed essentially as described.[9,24] The sensitivity of our method of detecting the IgH gene rearrangement has been previously assessed.[9]

MRD with patients' specific primers (ASO PCR)

VDJ sequence. VDJ gene rearrangement amplification was performed with a panel of VH family specific primers, together with a JH consensus primer. Seven amplifications were performed for each patient in order to determine the VH segment involved in the myeloma VDJ gene rearrangement. The reaction mixture (50 µL) contained 200 mmol/L dNTPs, 1 X PCR buffer (10 mM βME, 6.7 mM EDTA pH 8, 67 mM Tris pH 8.8, 170 mg/mL BSA), 7.7 mM $MgCl_2$, 50 pmol of each primer, 2% DMSO and 0.3 U Taq DNA polymerase (Boehringer Mannheim, Malta-Italy). Thirty cycles of amplification were performed, consisting of denaturation at 95°C for 30 sec, annealing at 61°C for 40 sec and extension at 72°C for 50 sec, followed by a 7 min final extension at 72°C. A 15 µL aliquot was analyzed by ethidium bromide staining 3% agarose gel: a single discrete 300 bp band was obtained for each patient. A 30 µL aliquot of amplification product, corresponding to the VH family-specific gene rearrangement, was loaded on a 1.25% low melt preparative grade agarose gel (BioRad, Segrate, Italy). The ~300 bp band was excised from the gel and purified with a Gel Nebulizer Micropure™ Separator (Amicon, Italy), according to the manu-

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Table 1a. Patients' characteristics.

Characteristics	*no. of patients*
Median age in years (range)	51.5
Sex (M/F)	5/5
Tumor stage*	
I	4
II	3
III	3
M component	
IgG	6
IgA	3
BJ	0
non secretory	1
Light chain	
λ	3
κ	6
non secretory	1
Renal insufficiency	
yes	/
no	10
Previous therapy	
Alkylating agents	2
VAD or VAD-like therapy	3
VAD + alkylating agents	4
α-IFN alone	/
radiotherapy alone	1

**according to Durie-Salmon classification.*

facturer's instructions. An aliquot of purified DNA was directly sequenced with the family-specific VH primer, using the Thermo Sequenase DNA cycle-sequencing kit (Amersham Italia, Italy), according to the manufacturer's instructions. Sequence analysis was performed using PC-GENE software (IntelliGenetics). Patient-specific primers were designed from the sequence information of the CDR-II and CDR-III regions in order to generate clonal patient-specific amplification (Table 1b).

Molecular follow-up of MRD with patients' specific primers. Follow-up studies for the detection of MRD were performed on bone marrow specimens, taken at various times after BMT. The minimum follow-up period for all patients was 3 months, the maximum 48 months. Clonally expanded B-cells were detected by amplifying 1 µg of DNA, using the patient's specific CDR-II and CDR-III primers. Fifty cycles of amplification were performed, consisting of denaturation at 96°C for 30 sec, annealing at the best tested temperature for 30 sec and extension at 72°C for 40 sec, followed by a 7 min. final extension at 72°C. The reaction mixture (50 µL) contained 200 mmol/L dNTPs, 1X PCR buffer (500 mM KCl, 100 mM Tris, pH 8.3), 2.5 mM $MgCl_2$, 50 pmol of each patient-specific primer and 1 U of AmpliTaq Gold™ (Perkin Elmer, Milan, Italy). A 15 µL aliquot was analyzed on agarose gel as described above. A 150 bp ca. band was obtained in each patient analyzed. Sensitivity of each set of primers was checked on serial dilutions of patient's DNA from initial marrow samples in an appropriate amount of DNA from normal peripheral blood. Using this CDR-III ASO PCR assay we were able to increase the sensitivity of detection of MM cells in 10^{-5} to 10^{-6} dilutions.[9]

Results

Clinical data and mobilization of tumor cells and hematopoietic progenitor cells

The clinical characteristics of the patients are reported in Table 1a. All patients received one or more lines of treatment before high dose Cy. None of them was in CR at the time of the study. Of the 10 patients, 2 had been treated with a median of 6 cycles of an alkylating agent-containing regimen (mostly melphalan and prednisone), 3 patients had received 4 cycles of VAD therapy (vincristine, doxorubicin, and dexamethasone), and 4 individuals had received both. One patient received radiotherapy alone (Table 1a). The circulating plasma cells levels were recorded before initiation of PBSC mobilization and have been already reported.[9] Kinetic analysis showed a pattern of tumor cell mobilization similar to that of normal hematopoietic progenitor cells, with a maximum peak falling within the optimal time period for the collection of PBSCs.

Ex vivo purging of tumor cells by positive selection of CD34+ cells

Contamination of neoplastic plasma cells and B cells in 8 patients has been reported.[9]

All 10 MM patients had their circulating CD34+ cells purified by the Ceprate SC concentrator. After positive selection a reduction of 99.7±0.2% of plasma cells was documented by microscope immuno-

Table 1b. Sequences of the clonal CDR-III region of the IgH gene of the two MM patients undergoing molecular follow-up with patient-specific primers. VH-family = family specific VH segment used in the V-D-J rearrangement. Primers (sense and antisense) are given as 5'-3' sequences. Annealing temperatures are reported.

No.	*Name*	*VH family*	*Primer sense (5'-3')*	*CDR-III sequences (5'-3')*	*Primer antisense (5'-3')*	*Annealing temp.*	*Size of PCR product*
1	ZERE	VH3	CGG GAT TTC ATC TGA TGG AAC	VH3 GCC TCC TAC CAT TTT GCA ACA GCG TTC T JH3	TGT TGC AAA ATG GTA GGA GGC	61°C	169 bp
2	MIRO	VH3	ATT ACT AGT AGT ACT TTG	VH3 GTG GGA GCT TGG G JH4	CTC CCA AGC TCC CAC	50°C	153 bp

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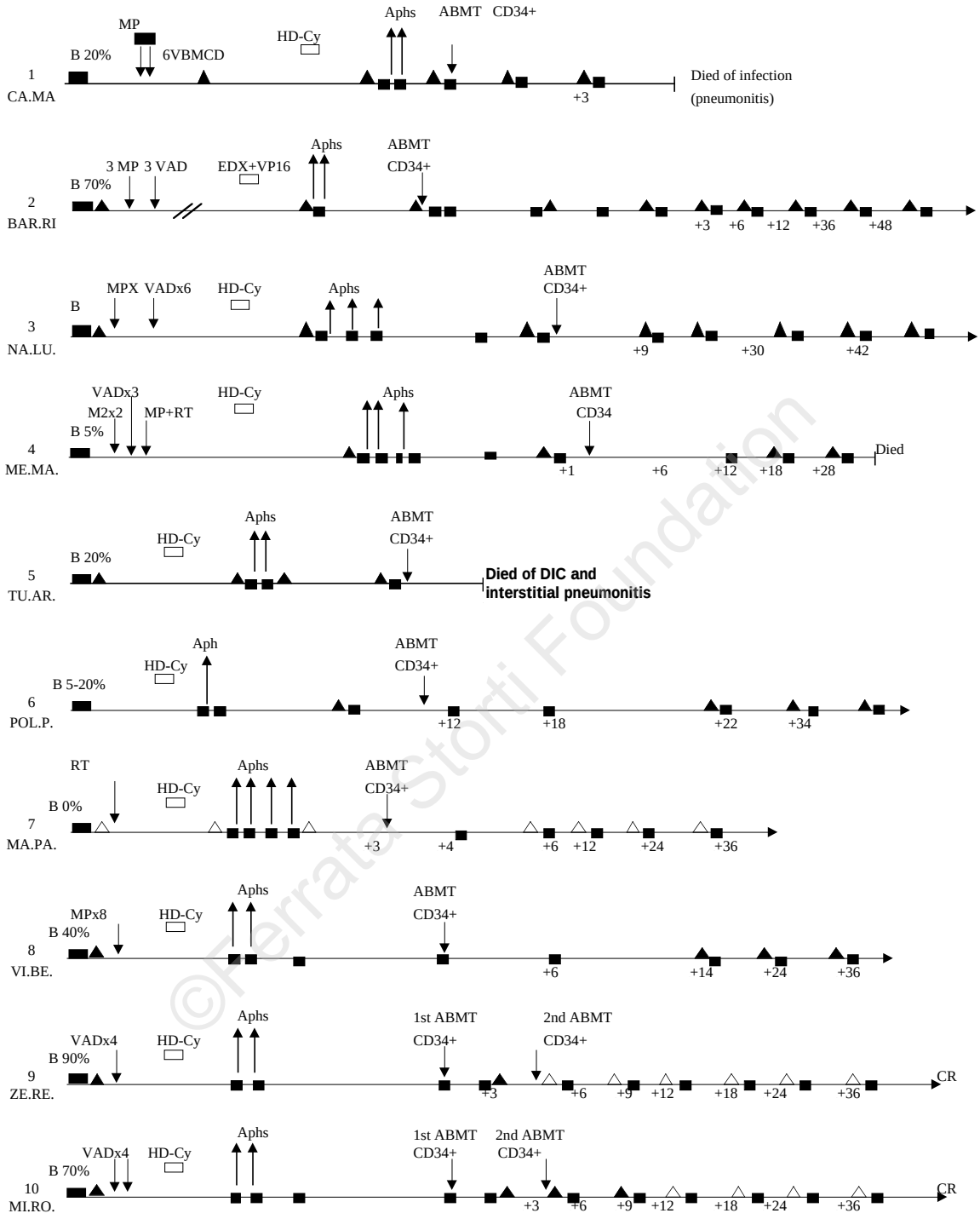

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Figure 1. Schematic representation of clinical and molecular follow-up of the MM patients. Each patient is represented by a line: numbers under the lines show the months of follow-up.

ABMT = autologous bone marrow transplantation, CR = complete hematologic remission, HD-Cy = high dose cyclophosphamide. Open rectangle over the line represents HD-Cy. MP, MPX, VBMCD, VAD represents different chemotherapy schedules. The number before the chemotherapy represents the number of cycles of chemotherapy. RT= radiotherapy. Aphs= apheresis. Full and open triangles on the line represent IF positive and negative analyses, respectively. Full and open boxes on the line represent PCR CDR-III (ASO for #9 and 10) positive and negative analyses, respectively. The numbers followed by a plus mark (+) represent months of clinical follow-up after ABMT.

fluorescence and flow cytometry analysis. Interestingly, the percentage of plasma cells decreased after positive selection from 0.7±0.4% to 0.1±0.07% ($p<0.3$). This calculation indicated that tumor cells did not merely behave as *innocent bystanders* during the $CD34^+$ cell purification process, and that approximately 1-log depletion of myeloma cells was caused by the procedure. An additional 2-log purging resulted from the overall recovery of only 0.8% of MNCs.[9]

PBSC processing data, engraftment results, recovery and clinical outcome

The recovery of hematopoietic progenitor cells of most of our patients has already been reported along with engraftment and supportive-care data.[9] All MM patients achieved granulocyte engraftment in a median of 10 days and an unsupported platelet count $>20\times10^9$/L in a median of 15 days, respectively ($p>0.1$). The median time to reach $>50\times10^9$ Plt/L was 12 days for $CD34^+$ cells. One patient (#1) did not achieve platelet recovery (she had been reinfused with less then 2×10^6 $CD34^+$ cells/kg), and she died in the peritransplant period from interstitial pneumonia. Other clinical parameters, such as the duration of hospitalization, transfusion requirement, number of febrile days, documented infections and use of intravenous antibiotics were not different between this group and another group of MM patients transplanted with unmanipulated PBSC.[9] No patient received backup PBSCs or required platelet transfusion after discharge from hospital. Two patients (#9 and 10) were submitted to a second ABSCT and reinfused with $CD34^+$ cells in the same manner. Similar reconstitution results were obtained as in the first ABSCT. With a median time from reinfusion of 36 months (range 3-48), we have not thus far observed any late graft failure in patients who received purified $CD34^+$ cells. Seven patients are alive in hematologic remission: four of them had serum M protein assessable by immunofixation analysis, and two turned from serum IF positivity to negativity after 6 and 12 months from ABSCT, respectively. One was not secretory and three patients died, one from disease progression and body failure and two from interstitial pneumonia (Figure 1). All but one of the patients reinfused with $CD34^+$ cells are currently being treated with 3 U/m^2 of α-IFN (3 times a week) as maintenance therapy.

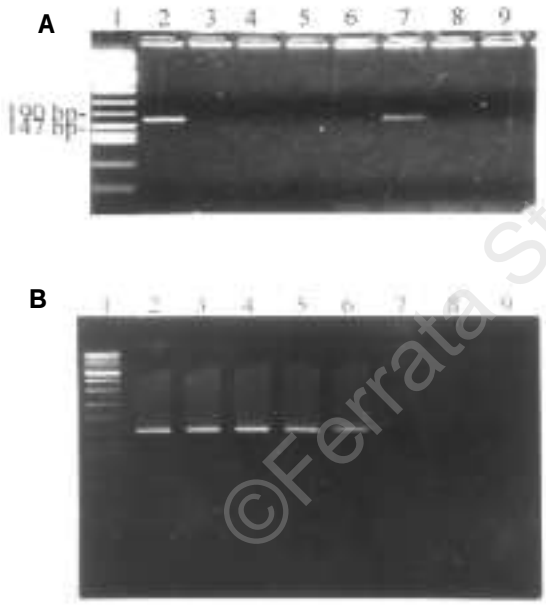

Figure 2. Molecular follow-up of patients #9 and #10 with patient-specific primers.
2a: Specificity of patient-specific PCR assay.
1 = molecular weight marker VIII (Boehringer Mannheim); 2 = patient #9 specific band, obtained with her patient-specific primers (PCR^+); 3 = patient #10 DNA amplified with patient #9 specific primer (PCR^-); 4 = negative control (DNA of a normal individual) (PCR^-); 5 = dH_2O (PCR^-); 6 = patient #9 DNA amplified with patient #10 specific primer (PCR^-); 7 = patient #10 specific band, obtained with his patient-specific primers (PCR^+); 8 = negative control (PCR–); 9 = dH_2O (PCR^-).
2b: Sensitivity of patient #9-specific PCR assay at DNA level.
1 = Molecular weight marker VIII (Boehringer Mannheim); 2 = 10^{-1} dilution assay; 3 = 10^{-2}; 4 = 10^{-3}; 5 = 10^{-4}; 6 = 10^{-5}; 7 = 10^{-6}; 8 = 10^{-7}; 9 = dH_2O

Evaluation of MRD on aphereses by DNA amplification of the CDR-III region of the IgH gene with consensus primers

Consistent with immunofluorescence studies, MRD was observed after stem cell purification in 8/10 patients' samples which were evaluated by PCR analysis of IgH gene rearrangement, with consensus primers as reported.[9] A schematic representation of clinical, laboratory and molecular outcomes is shown in Figure 1. The original clonal bands, identical in size to those observed in diagnostic BM samples were also found in each leukapheresis. In two patients (#9 and 10), the leukaphereses were only evaluated with patients' specific primers.

Molecular follow-up by PCR analysis of IgH gene rearrangement by patient specific primers

Patient-specific primers were generated to study the patient-specific IgH gene rearrangement in 2 patients (#9 and 10) who were IF negative 12 months after ABSCT (Figure 1). We were able to determine the CDR-III coding region by direct sequencing of PCR product generated by family specific primers. We generated the MM patients' specific primers (based on the CDR-II and CDR-III regions), and verified their patient-specificity (Figure 2a). We also assessed the sensitivity of our PCR assay at DNA level, which for each patient could detect at least one neoplastic cell diluted in 10^{-5}-10^{-6} negative cells (Fig-

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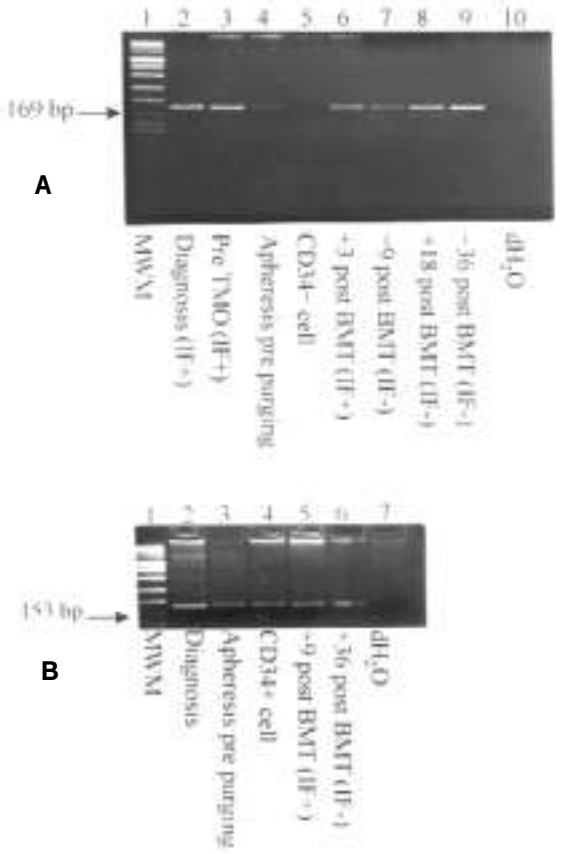

Figure 3. Molecular follow-up of patients #9 and 10 with patient-specific primers.
3a: specificity of patient-specific PCR assay.
1 = molecular weight marker VIII (Boehringer Mannheim); 2 = diagnosis (IF^+); 3 = pre-TMO (IF^+); 4 = apheresis pre-purging; 5 = $CD34^+$ cell; 6 = +3 post-BMT (IF^+); 7 = +9 post-BMT (IF^-); 8 = +18 post-BMT (IF^-); 9 = +36 post-BMT (IF^-); 10 = dH_2O.
3b: sensitivity of patient-specific PCR assay at DNA level.
1 = molecular weight marker VIII; 2 = diagnosis; 3 = apheresis pre-purging; 4 = $CD34^+$ cell; 5 = +9 post-BMT (IF^+); 6 = +36 post-BMT (IF-); 7 = dH_2O.

ure 2b). We found that although the stem cell purified fraction ($CD34^+$ cells) from patient #9 was negative for clonal rearrangement and that of patient #10 was positive, during follow-up both patients showed the presence of neoplastic cells in all the samples, even though they had become IF negative (Figure 3a and 3b).

Discussion

Circulating hematopoietic stem cells (PBSC) are generally believed to be involved by myeloma less frequently than BM harvests.[25] The use of PBSC in transplantation offers several advantages over BM stem cells, including a faster recovery of hematopoiesis. For these reasons, PBSC transplantation is being used in many centers for the treatment of MM patients after myeloablative therapy.[5,6] Indirect purging of neoplastic cells provided by positive selection of hematopoietic $CD34^+$ cells, has recently been proposed.[9,26,27] Our study confirms that the concomitant mobilization of plasma cells and hematopoietic progenitors is detectable at molecular level. Leukapheresis products were always contamined by myeloma cells. Other authors[12-25,28] showed similar contamination of PBSC collections by myeloma cells. The biological and prognostic significance of tumor cells present in PBSC collections is still unknown, as relapse may be caused either by re-growth of residual clonogenic cells *in vivo*, or by reinfused malignant cells.[29] Thus, removal of myeloma cells from PBSC autografts by positive selection of hematopoietic $CD34^+$ cells could have biological effects on PBSC outcome.[6,30]

Since the CD34 antigen is known not to be expressed on the surface of mature plasma cells in MM,[31,32] anti-CD34 antibodies may be clinically useful for isolating hematopoietic precursors for transplantation in MM patients with a reduced but not eliminated neoplastic clone.[26] It has been reported that in collections of 99.99% pure $CD34^+$ cells, obtained after using the combination of two methods of purification, IgH gene clonal rearrangement was not detectable by means of PCR based technology.[26, 32] We confirm this observation (in case #9 of our series), which reinforces the validity of using $CD34^+$ enriched cells as a source of possible uncontaminated cells for ABMT.[33] Reinfusion of autologous $CD34^+$ cells has been shown to reconstitute normal hematopoiesis in MM and other cancer patients treated with high-dose chemotherapy.[9]

The clinical study and follow-up of these MM patients also provides evidence that purified $CD34^+$ progenitors are capable of restoring bone marrow function in MM patients in the early phase of ABMT, as well as in the subsequent months. To date, no late granulocyte or platelet engraftment failure has been observed in any of our surviving patients, despite maintenance treatment with α-IFN (median follow-up: 36 months), and our study confirms that such a $CD34^+$ enriched cell fraction is capable of reconstituting stable hematopoiesis. Thus, the rapid and sustained engraftment, together with the documented depletion of tumor cells, shows that positive selection produces an MM-purging effect without apparent loss of engraftment potential.

As far as regards clinical outcome, two of the ten patients died of infection (pneumonia) in the first year after ABMT. We think that this complication is probably more related to the ABMT conditioning regimen (including TBI), than to the immunosuppression associated with the transplantation of the purified $CD34^+$ cells. In fact, we and others have recently reported that the immunologic reconstitution of patients transplanted with $CD34^+$ cells does not significantly differ from that of past patients who received unmanipulated stem cells.[34] Seven patients are in hematologic remission and two of them have

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no IF evidence of disease.

Concerning molecular follow-up, we have recently developed an assay using patient-specific primers[12] in order to achieve better specificity and if possible higher sensitivity for our PCR based IgH clonal rearrangement assay. Our present patient-specific PCR analysis at RNA level detects one IgH clonal rearranged cell diluted in 10^5-10^6 non-neoplastic cells with absolute patient-specificity, even though some discrepancies in sensitivity could be present due to different annealing temperatures, length of primers, etc. We applied this PCR strategy in the two patients who achieved IF negativity, and we showed that both remained PCR positive for IgH clonal rearrangement during follow-up. As the contribution of contaminating MM cells in PBSC collection to clinical relapse is currently unknown, it is difficult to assess their role in MRD.[28,32]

We conclude that positively selected $CD34^+$ cells provide a cell suspension that can be safely used as supportive therapy for patients undergoing a myeloablative conditioning regimen. This procedure allowed restoration of persistently normal hematopoiesis in the majority of our MM patients.

Contribution and Acknowledgments

GM was the principal investigator: he designed the study and was responsible for ethical approval of the program; MC was responsible for funding and direct supervision. CT and MA set up the PCR procedures and drafted the paper. MB and MRM developed and carried out the cryopreservation procedures and flow cytometry assay. NT and AdV were responsible for cytogenetic studies, ST, MC and RML critically revised the manuscript and gave the final approval for its submission.

The order of authorship was made on the basis of the contributions given to the study.

We thanks P. Corradini and C. Voena from Institute of Hematology, University of Turin, Italy, for the PCR assays, and for their helpful suggestions.

Funding

This study was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC), by the Italian CNR no. 98.00526.CT04, by "AIL 30 Ore per la Vita", and MURST 40% target projects.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received October 26, 1998; accepted March 4, 1999.

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Haematologica 1999; 84:405-412 original paper

The flow cytometric pattern of CD34, CD15 and CD13 expression in acute myeloblastic leukemia is highly characteristic of the presence of PML-RARα gene rearrangements

ALBERTO ORFAO*, MARÍA CARMEN CHILLÓN, AGLAÊ Mª BORTOLUCI, MARÍA CONSUELO LÓPEZ-BERGES, RAMÓN GARCÍA-SANZ, MARCOS GONZALEZ, MARÍA DOLORES TABERNERO,* MARÍA ANTONIA GARCÍA-MARCOS, ANA ISABEL RASILLO,* JESÚS HERNÁNDEZ-RIVAS, JESÚS F. SAN MIGUEL

*Servicio de Hematología, Hospital Universitario Salamanca; *Servicio General de Citometría, Centro de Investigaciónes del Cáncer de Salamanca and Departamento de Medicina, Universidad de Salamanca, Salamanca, Spain*

ABSTRACT

Background and Objective. **Rapid identification of AML patients carrying the t(15;17) translocation for treatment decision-making is currently made on the basis of morphologic screening. However, the existence of both false positives and negatives highlights the need for more objective methods of screening AML cases and further molecular confirmation of the t(15;17) translocation.**

Design and Methods. **In the present study we analyzed a total of 111 AML cases in order to investigate whether immunophenotyping based on the assessment of multiple-stainings analyzed at flow cytometry could improve the sensitivity and specificity of morphologic identification of acute promyelocytic leukemia (APL) carrying the t(15;17) translocation. FISH analysis was used as a complementary technique for cases in which morphology and molecular biology yielded discrepant results.**

Results. **Concordant results between morphology and RT-PCR were found in 102/111 (91.8%) cases: 34 patients had M_3/PML-RARα$^+$ and 68 non-M_3/PML-RARα$^-$ disease. Nine cases showed discrepants results. Multivariate analysis showed that the best combination of immunologic markers for discriminating between M_3/PML-RARα$^+$ and non-M_3/PML-RARα$^-$ cases was that of the presence of heterogeneous expression of CD13, the existence of a single major blast cell population, and a characteristic CD34/CD15 phenotypic pattern (p<0.02). A score system based on these parameters was designed, and the 34 M_3/PML-RARα$^+$ cases showed a score of 3 (presence of the 3 phenotypic characteristics). In contrast, only 1 out of the 68 (1.3%) non-M_3/PML-RARα$^-$ cases had this score, most of these latter cases (53/68, 78%) scoring either 0 or 1. Therefore, among these cases, immunophenotyping showed a sensitivity of 100% and a specificity of 99% for predicting PML/RARα gene rearrangements. Of the 9 cases in which morphology and molecular biology results were discrepant, four cases displayed M_3 morphology without PML/RARα rearrangements by RT-PCR. In only one of these 4 cases did the immunophenotype score 3, this being the only FISH positive case. From the remaining five discrepant cases (non-M_3 morphology while positive for PML/RARα) two cases had a phenotypic score of 3 and were FISH positive while the other three were negative by FISH. Upon repeating RT-PCR studies, two of these latter three cases became negative.**

Interpretation and Conclusions. **Our results show that immunophenotyping may be of great value for quick screening of APL with PML/RARα rearrangements.**

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Key words: acute myeloblastic leukemia, t(15;17)

Correspondence: Alberto Orfao, MD, PhD, Servicio General de Citometría, Hospital Universitario Salamanca, Paseo de San Vicente, 58-182, 37007 Salamanca, Spain.
Phone: international +34-923-291375 – Fax: international +34-923-294624 – E-mail: orfao@gugu.usal.es

Acute myeloblastic leukemia (AML) with translocation t(15;17) which involves the PML and RARα genes, represents a well-defined subgroup of AML-acute promyelocytic leukemias (APL) which benefits from a specific therapeutic strategy based on the combination of all-transretinoic acid (ATRA) plus chemotherapy.[1-6] Because of this, once a diagnosis of AML has been established one of the most important goals is to assess whether or not the leukemic cells do carry the t(15;17) translocation. Detection of t(15;17) can be currently performed using conventional cytogenetics,[7] FISH analysis[8] and molecular biology using the reverse transcriptase polymerase chain reaction (RT-PCR) and Southern-blot hybridization.[9] However, from the clinical point of view these techniques still have several limitations the most notable being that they are time-consuming and thus treatment must be initiated without their results; furthermore, some of these techniques are not available in all centers. It would, therefore, be of great value to have other approaches for rapid identification of patients carrying the t(15;17) translocation.

So far, the only such approach is morphology since there is a very good correlation between M_3 morphology and the t(15;17) translocation. However, the subjectivity of morphology analysis produce false pos-

itive diagnoses and false negative results. Additionally, although t(15;17) translocation is one of the most frequent structural chromosome abnormalities in AML patients it is only present in a relatively low proportion of AML cases, its incidence ranging from 6% up to 38% depending on the geographical area.[10] Because of this relatively low incidence, molecular confirmation of the PML/RARα gene involvement is not generally investigated in all AML cases but on the select cohort of patients that are morphologically diagnosed as having M_3 leukemias.[1,4,5,6,11,12] Accordingly, the availability of other more objective methods of screening APL leukemias for further molecular confirmation would be of great value.

For more than 10 years a clear association between FAB M_3 morphology and the immunophenotype of AML blast cells has been established. Blast cells in APL usually co-express the myeloperoxidase (MPO), CD9, CD13 and CD33 myeloid-associated markers in the absence of reactivity for HLA-DR.[13-15] In spite of this association, AML patients displaying this phenotype still form a heterogenous group of patients including a substantial proportion of patients who do not display the t(15;17) translocation; moreover, a small proportion of APL cases are HLA-DR^+.[13-16] Due to these limitations immunophenotyping of AML blast cells has been considered as a secondary diagnostic tool with respect to morphology for the identification of cases carrying the t(15;17) translocation and its use has been almost exclusively restricted to supporting the morphologic diagnosis of M3 variants (hypogranular morphology).[17-19] In recent years the availability of antibody reagents directed against the PML protein has shown that both the wild PML protein and the PML-RARα fusion protein display different immunocytochemical patterns of staining which directly correlate with the molecular studies.[20-23] However, discrepant results have been reported regarding the characteristic intracellular distribution of the fused nuclear[21,22] versus cytoplasmatic[20-23] protein. Moreover, Falini *et al.*[22] showed that in up to 8% of the AML cases analyzed no staining was observed with the anti-PML monoclonal antibody, these patients being considered as false negative cases with respect to the expected PML reactivity.

In parallel to reagent development important advances have occurred in recent years regarding the immunophenotypic characterization of leukemic cells as a result of the possibility of performing multiple stainings which allow the identification of leukemic cells by distinguishing them from their normal counterparts, and the specific analysis of leukemic cell differentiation.[18,19,24] In spite of this, to the best of our knowledge no study has been performed in which the sensitivity and specificity of multiparametric immunophenotyping has been compared with conventional morphologic criteria for the identification of cases carrying the t(15;17) translocation.

The aim of the present study was to evaluate the immunophenotypic characteristics of AML patients based on multiple stainings analyzed by flow cytometry in order to assess whether or not immunophenotyping improves the sensitivity and specificity of morphology and provides a useful tool for the screening of cases in which the t(15;17) translocation should be systematically searched for by molecular techniques.

Design and Methods

Patients

A total of 111 patients (104 adults and 7 children) whose BM samples were stored in the reference laboratory of the University Hospital of Salamanca were included in the present study. All patients had an unequivocal diagnosis of *de novo* AML based on morphologic, cytochemical[25] and immunophenotypic criteria.[17] Sixty-eight patients were male and 43 were female with a mean age of 51±21 years; the range was from 6 to 87 years old. Two experts independently assessed FAB morphology. All cases were studied at diagnosis.

Immunophenotyping studies

In all cases immunophenotyping studies were performed at diagnosis on erythrocyte-lysed whole bone marrow (BM) samples upon staining with monoclonal antibodies directly conjugated with fluorochromes. Antigen expression was analyzed on a FACSort flow cytometer (Becton Dickinson, San José, CA, USA) using double and triple-stainings with the following combinations of fluorochrome-conjugated monoclonal antibodies (fluorescein isothiocyanate [FITC], phycoerythrin [PE] and either peridin chlorophyll protein [PerCP] or the PE/cyanide 5 [Cy5] fluorochrome tandem) directed against surface antigens: CD15/CD117/CD34, CD15/CD33/CD34, CD15/CD34/HLA-DR, CD34/CD38/CD19, CD34/CD56/CD33, HLA-DR/CD33/CD13, CD7/CD13/CD19, CD65/CD11b/CD4, CD2/CD14/CD13, CD61/glycophorin A/CD45, CD10/CD5/CD20 and CD71/CD11b. In addition the expression of MPO, CD79a and CD3 was also explored at the cytoplasmic level.

Briefly, BM samples were obtained and immediately diluted in phosphate buffered saline (PBS) containing K_3 EDTA as anticoagulant in a 1/1 (vol/vol) proportion. Afterwards, for surface antigenic stainings, 200 µL of PBS-diluted BM samples, containing between 0.5 and 1×10^6 nucleated cells were placed in each tube and incubated with the appropriate combination of monoclonal antibodies for 10 minutes in the dark (room temperature). Once this incubation period was finished, 2 mL of FACS lysing solution (Becton/Dickinson) diluted 1/10 (vol/vol) in distilled water were added to each tube and after vigorous vortexing another incubation for 10 minutes in the dark (room temperature) was performed. Cells were then centrifuged (5 minutes at 540 g), washed once in 2 mL of PBS/tube (5 minutes at 540 g) and resuspended in 0.5 mL/tube of PBS.

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For the staining of cytoplasmic antigens (MPO, CD79a and CD3) the Fix & Perm reagent from Caltag Laboratories (San Francisco, CA, USA) was used. Briefly, 50 µL of sample were incubated with 100 µL of solution A from the Fix & Perm reagent for 15 minutes (room temperature). Afterwards, cells were washed once in 2 mL of PBS/tube and resuspended in 100 µL of an erythrocyte-lysing, leukocyte-permeabilizing solution (solution B from the Fix & Perm reagent). In addition, 10 µL of anti-MPO-FITC, anti-CD79a-PE and anti-CD3-PE/Cy5 were added and cells incubated for another 15 minute period at room temperature. Afterwards, cells were resuspended in 2 mL of PBS/tube, centrifuged (5 minutes at 540 g) and resuspended in 0.5 mL of PBS.

The source and specificity of each monoclonal antibody used in the present study was as follows: CD34 (HPCA-2-PE, My10-FITC and HPCA-2 PerCP), CD15 (leu M1-FITC), CD33 (leu M9-PE), HLA-DR (anti-HLA DR-PerCP and anti-HLA DR-FITC), CD38 (leu 17-PE), CD56 (leu 19-PE), CD13 (leu M7-PE), CD7 (leu 9-FITC), CD2 (leu 5b-FITC), CD11b (leu 15-PE), CD14 (leu M3-PE), CD45 (HLE-1-PerCP), CD10 (CALLA-FITC), CD5 (leu 1-PE), CD71 (anti-transferrin receptor-FITC) and CD3 (leu 4-PerCP), were purchased from Becton/Dickinson; CD117 (95C3-PE) and glycophorin A (D2.10-PE) from Immunotech (Marseille, France); CD19 (SJ25C1-PE/Cy5),CD33 (4D3-PE-Cy5), CD13 (TUK 1-PE-Cy5), CD65 (VIM 2-FITC), CD4 (53.5-PE/Cy5), MPO (H435-FITC) and CD20 (HI47-PE-Cy5) were obtained from Caltag Laboratories and CD61 (Y2/51-FITC) and CD79a (HM57-PE) from Dako (Glostrup, Denmark).

Isotype-matched mouse non-specific immunoglobulins and a tube stained for the CD3-FITC, CD4-PE and CD8-PE-Cy5 antigens were used as negative and positive controls, respectively.

Data acquisition was performed on a FACSort flow cytometer (Becton/Dickinson) using the LYSIS II sofware program (Becton/Dickinson). A minimum of 15,000 events/tube from the total BM cellularity were acquired. In order to make results comparable between different days, careful instrument calibration and fluorescence compensation was performed using both CALIBRITE beads (Becton/Dickinson) and normal PB lymphocytes stained for CD3-FITC, CD4-PE and CD8-PE/Cy5 as described elsewhere.[26] The PAINT-A-GATE PRO software (Becton/Dickinson) was used for data analysis. Whenever necessary, further stainings were made in a second step in order to obtain specific information on the phenotypic characteristics of leukemic cells. The following information was specifically explored in leukemic cells for each of the antigens analyzed: presence or absence, fluorescence intensity and pattern of expression (homogeneous versus heterogeneous). A pattern of antigen expression was defined as heterogeneous if the cells occupied more then one logarithmic decade on the scale of fluorescence intensity. The presence of two or more major blast cell subpopulations was defined on the basis of the existence of phenotypically different leukemic cell subsets which each represented more than 25% of all neoplastic cells for any of the antigens analyzed except CD34.[26]

PCR amplification of PML/RARα transcripts

RNA was extracted from washed BM mononuclear cells by the guanidium thiocyanate method of Chomczynski and Sacchi.[27] Reverse transcription (RT) was performed on 1 µL of total RNA, after heating at 70°C for 10 minutes with random hexamers as the reaction primer. The reaction was carried out at 42°C for 1h in a 20 µL volume containing 200 U of Superscript II (Life Technologies Inchinnan, Scotland, UK) according to the manufacture's instructions. Subsequently, 5 µL of RT products were used for two-step PCR analysis according to the guidelines proposed by Biondi *et al*.[28] PCR conditions were as follows: reaction volume of 100 µL containing 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, and 10 mmol/L Tris HCl; pH 8.8; 200 µmol/L dNTP, 2.5 U of Taq DNA polymerase and 30 pmol of each primer. Primers used were M4, M2, R5 and R8, as previously described.[28]

PCR was performed in a Gene-Amp PCR System 9600 thermocycler (Perkin Elmer, Foster City, CA, USA). After an initial denaturation at 95°C for 5 minutes, denaturation, annealing and extension were carried out at 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, respectively, for a total of 30 cycles, with the last extension at 72°C lasting for 10 minutes. The first PCR was performed with the M4 primer (bcr 3 breakpoint) or M2 (bcr 1 or bcr 2 breakpoint) as 5' primers and the R5 as 3' primer. For the second-round PCR, the system used was the same as for the first round except that 5 µL of the first-PCR product were used instead of the RT product and the substitution of R5 primer for the nested primer (R8). Finally, 20 µL of the PCR product were electrophoresed on a 2% Nu Sieve agarose gel stained with ethidium bromide and visualized under UV light. Two negative controls (one with non-APL RNA and one without RNA) and one positive control APL sample were included in each experiment. The integrity of the RNA preparation was assessed by amplification of normal RARα as previously described by Borrow *et al*.[29] In order to exclude false positive or negative results, those samples with discrepancies between RT-PCR and morphology underwent to new RT-PCR assays using a different whole BM aliquot. Moreover, Southern blot analysis with the H18 and RARα-cDNA probes was carried out on these samples, as previously described.[30]

FISH studies

In those *de novo* AML cases in which discrepant results were found between morphology and the RT-PCR techniques, FISH analysis for the t(15;17) translocation was performed. For that purpose the LSI-PML/RARα dual color probe (Vysis Inc., Downers

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Grove, IL, USA) was used. The PML probe stained with spectrum orange begins in intron 7, a span of 5.8 Kb and extends centromericly on chromosome 15 approximately 180 Kb. The RARα probe begins less than 6 Kb 3' to intron 2 and extends approximately 400 Kb telomerically on chromosome 17. Accordingly, the combination of both probes covers the three break-point clusters (bcr 1, bcr 2 and bcr 3) within the region of 13 Kb of the PML gene on chromosome 15.

Prior to hybridization 1 µL of probe solution (Vysis) was mixed with 7 mL of LSI hybridization buffer (Vysis) and 2 µL of distilled water, centrifuged for 2 seconds and heated for 5 minutes in a 74±1°C water bath for denaturating purposes.

FISH analysis was performed on cells from BM samples prepared according to conventional cytogenetic techniques. Briefly, the slides containing fixed cells were immersed in a denaturating bath (70% formamide in 2x SSC buffer placed at 74±1°C) for 5 minutes and then dehydrated according to previously reported techniques[7,8] and dryed. Afterwards, slides were warmed to 45-50°C for 2 minutes and 10 uL of the denaturated probe-mix was applied to each slide. Hybridization was then allowed to take place by incubating the slide overnight (16 hours) in a pre-warmed humidified box placed in a 37°C incubator. Once this incubation period was finished, slides were washed 3 times (3×10 minutes) in 50% formamide in 2× SSC (pH=7.0) at 46°C. Another wash for 10 minutes in 2× SSC (pH =7.0) was then performed at room temperature. Finally, the slides were washed for 5 minutes (room temperature) in 2× SSC containing 0.1% of Tween 20 (Sigma, St. Louis, MO, USA). The slides were then allowed to dry and 10 µL of a counterstaining solution containing 75 ng/mL of DAPI (Sigma) and 20 mg/mL of 1,4 -diazobicyclo-2,2,2-octane (Sigma) used as an antifading agent were added to each slide.

Fluorescence signals were evaluated using a DMRX fluorescence microscope (Leica, Wetzlar, Germany) and a minimun of 200 cells/sample were analyzed including both interphase nuclei and metaphases. For all slides measured the number of unhybridized cells in the areas assessed was lower than 1% and only those spots with similar size, intensity and shape were counted. Cells with fusion or juxtaposed green and red signals were interpreted as positive for t(15;17).

Statistical methods

The relative frequencies of all the phenotypic variables included in the present study were calculated. Comparisons between groups were performed using the chi-square test and *p* values lower than 0.01 were considered to be associated with statistically significant differences (SPSS 5.0 Inc., Chicago, IL, USA).

For the assessment of the power of immunophenotypic criteria for discrimination between APL and non-APL cases (based on the presence or absence of PML/RARα transcripts) multivariate analysis was performed using a logistic regression model with the forward stepwise option and a probability comparison test (SPSS 5.0 Inc.). The immunophenotypic variables included in the multivariate analysis were those displaying statistical significant differences in the unvariate study.

Results

Of the 111 *de novo* AML cases included in the present study 38 displayed an M_3 morphology (34 cases were typical M_3 and four were considered hypogranular M_3 variants). Of the remaining 73 cases, 9 corresponded to M_0, 13 to M_1, 19 to M_2, 10 to M_4, 6 to M_4Eo, 8 to M_{5a}, 4 to M_{5b} and 4 to M_6. Initially, molecular studies showed the presence of the PML/RARα RNA transcript in a total of 39 out of the 111 patients (35%). Of them, 34 corresponded to AML cases with an M_3 morphology, and the remaining 5 cases were classified as having M_0 (2 cases), M_1 (one case) and M_2 (2 cases) leukemias. Accordingly, four different groups of patients could be established on the basis of the results obtained with both morphologic and molecular biology techniques. In the two major groups there was concordance between both methods: 1) M_3 cases being PML/RARα$^+$ (n=34); and 2) non-M_3/PML/RARα$^-$ cases (n=68); the remaining two groups included cases in which morphology and RT-PCR studies showed discrepant results; 3) M_3 morphology with negativity for the PML/RARα transcripts (n=4); and 4) non-M_3/PML-RARα$^+$ AML cases (n=5). RT-PCR studies were repeated in all the cases from these latter two groups of patients confirming the initial findings in all cases except for one M_0 and one M_1 patient who were initially PML/RARα$^+$ but became negative in the second and third analyses.

For the analysis of the immunophenotypic characteristics of the 111 AML patients, we first divided the series into two groups: the M_3/PML-RARα$^+$ and non-M_3/PML-RARα$^-$ cases (Table 1). In the former group the leukemic blast cells showed the following common characteristics: 1) homogeneous expression of CD33-PE in all blast cells (82% of the cases) (Figure 1B); 2) reactivity for CD13-PE in all leukemic cells but with a heterogeneous pattern of expression (100% of cases) (Figure 1C); 3) a singular pattern of expression for the CD34-PE/CD15-FITC antigens in which leukemic cells lose CD34 before they acquire CD15 expression, and the blast cells never acquire high levels of CD15 (100% of the cases) (Figure 1D and E); 4) absence of reactivity for HLA-DR-FITC (91% of the patients) (Figure 1B) and 5) presence of a single major blast cell population which may be defined by these antigens (100% of the cases). Interestingly, in the non-M_3/PML-RARα negative cases the incidence of each of the five immunophenotypic characteristics mentioned above was significantly lower ($p<0.00001$) (Table 1).

Multivariate analysis showed that the best combi-

Table 1. Comparative analysis of the immunophenotypic characteristics of M3 / PML-RARα+ and non-M3 / PML-RARα- AML cases: results of the uni- and multivariate studies.

	M_3/ PML-RARα+ n=34	*non-M_3/ PML-RARα- n=68*	*uni-variate analysis p value*	*multi-variate analysis p value*
Typical CD34/CD15 pattern	100%	21%	<0.00001	<0.00001
CD13+ heterogeneous	100%	34%	<0.00001	<0.00001
One major blast cell subset	100%	56%	<0.00001	<0.002
HLA-DR-	91%	24%	<0.00001	<0.03
CD33+ homogeneous	82%	34%	<0.00001	<0.19

nation of variables to discriminate between M_3/PML-RARα+ and non-M_3/PML-RARα- cases was that of the CD34/CD15 phenotypic pattern (p<0.00001), the expression of CD13 (p<0.00001) and the presence of a single major blast cell subset (p<0.002) (Table 1). When a score system based on these three parameters was applied to the two major groups of AML patients established on the basis of the morphologic (M_3 versus non-M_3) and molecular results (PML-RARα+ versus PML-RARα-) we found that all 34 M_3/PML-RARα+ cases had a score of 3 while only 1 out of the 68 (1.3%) non-M_3/PML-RARα- cases had this score. Most of the other cases (53/68, 78%) scored either 0 or 1 (Table 2). Therefore among these cases, immunophenotyping had a sensitivity of 100% and a specificity of 99% for predicting PML/RARα gene rearrangements. Within the 9 cases in which morphology and molecular biology were initially discrepant, four cases displayed M_3 morphology without PML/RARα gene rearrangements by RT-PCR (Table 2). In only one of these 4 cases was the score 3. Of the five cases with non-M_3 morphology which were PML/RARα+, two cases had a phenotypic score of 3 (Table 2). Table 3 shows the results obtained with the FISH technique for the analysis of the t(15;17) translocation in all these 9 cases with discrepant morphologic and molecular findings. As shown in it, FISH studies were positive only in those three cases with an immunophenotypic score of 3 while the remaining 6 cases were negative. As mentioned earlier, in all these 9 cases molecular biology studies using RT-PCR were repeated using different aliquot samples. In these repeated experiments, only three out of the 5 non-M_3/PML-RARα+ patients remained constantly positive while the other two cases became negative. These latter two cases were patients with an immunophenotypic score of 1 (Table 3). In contrast, the four M_3/PML-RARα- cases were repeatedly negative for the PML-RARα transcript, but one of them, with a score of 3 by immunophenotyping, showed a rearranged RARα gene by Southern blot analysis.

Discussion

At present, BM morphology is still commonly used as the only diagnostic tool for treatment decision-making in APL although the accuracy of morphology

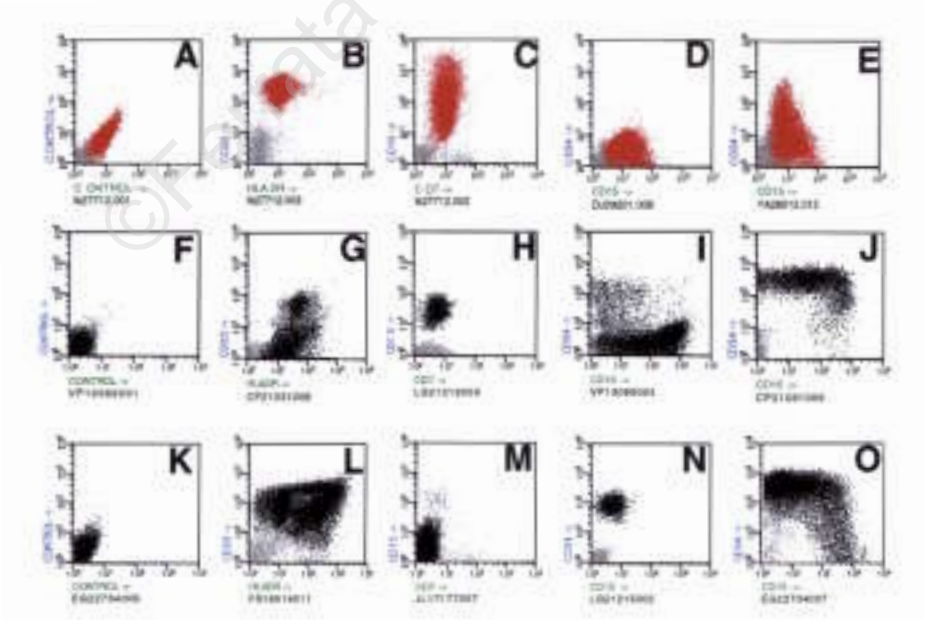

Figure 1. Characteristic immunophenotypic features of M_3 / PML-RARα+ AML blast cells (red dots) as compared to other PML-RARα- cases (black dots). Expression of CD33-PE and HLA-DR-FITC (B), CD13-PE (C) and pattern of blast cell differentiation for the CD34-PE / CD15-FITC antigens in two different patients (D&E). Panels A and F show an isotype-matched negative control: note the increased baseline fluorescence levels displayed by the leukemic blast cells of an M_3 / PML-RARα+ case.

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Table 2. Immunophenotypic score of AML cases grouped according to the morphological diagnosis and RT-PCR studies for the PML-RARα gene rearrangements.

Score	M_3/ PML-RARα$^+$	M_3/ PML-RARα$^-$	non-M_3/ PML-RARα$^+$	non-M_3/ PML-RARα$^-$
0	–	1	–	15
1	–	1	2	38
2	–	1	1	14
3	34	1	2	1
Total cases	34	4	5	68

Table 3. AML cases with discrepant morphology and RT-PCR results: immunophenotypic score, FISH analysis and results of confirmatory PCR studies.

	Immunophenotypic score	FAB	FISH	Confirmatory PCR
M3/PML-RARα$^-$				
Case 23308	0	classic M_3	–	–
Case 26810	1	classic M_3	–	–
Case 20968	2	classic M_3	–	–
Case 26584	3	classic M_3	+	–*
non M3/PML-RARα$^+$				
Case 18711	1	M_1	–	–
Case 21339	1	M_0	–	–
Case 21303	2	M_0	–	+
Case 21365	3	M_2	+	+
Case 28012	3	M_2	+	+

**positive by Southern-blot hybridization.*

is considered to be insufficient.[9,22] Thus, demonstration of a PML-RARα rearrangement as a result of the t(15;17) translocation is mandatory due to the sensitivity of APL to ATRA treatment.[1-6] As a matter of fact a recent study by Tallman *et al.*[12] showed that 40% of the cases that were diagnosed as M_3 leukemia and in which ATRA treatment was started were then shown to be PML-RARα$^-$, which would correspond to false positive cases by morphology, and subsequently these patients, had to interrupt ATRA treatment. In a Spanish trial, the reported incidence of false positive APL based on morphology (M_3) was 14%.[11] To the best of our knowledge, few studies using molecular analysis have been performed to assess the incidence of false negative cases by morphology, in order to exclude, among the non-M_3 leukemias, the presence of some PML-RARα$^+$, also showing the existence of false negative cases by morphology.

In the present study, the simultaneous assessment of BM morphology and the PML-RARα translocation in a group of 38 M_3 leukemias and 73 consecutive AML cases with a non-M_3 morphology showed that, although in most cases (91.8%) agreement is observed between both methods, there is also a small proportion of M3 cases (10.5%) which do not have the PML-RARα transcript, as well as cases with non-M_3 morphology that are PML-RARα$^+$ based on diagnosis by RT-PCR techniques (6.8% of all the non-M_3 cases). This supports the notion[22] that not only false positive but also false negative cases may exist if morphology is taken as the single tool for treatment decision making and to select cases who are candidates for further molecular studies.

For a long time APL has been shown to display a characteristic phenotype which has been mainly associated with the co-expression of pan-myeloid markers such as CD13 and CD33 in the absence of reactivity for the HLA-DR antigen.[13-16] However, both HLA-DR$^+$ APL and HLA-DR$^-$ AML displaying morphologic characteristics distinct from those of M_3 leukemias have been shown to exist, which suggests that immunophenotyping has a limited value as a primary diagnostic tool in APL.[17-19,31]

However, nowadays, the immunophenotypic characterization of blast cells should no longer be based merely on the presence or absence of an antigen, since multiparametric analysis using multiple simultaneous stainings analyzed by sensitive flow cytometric methods also provides information on the pattern of antigen expression and may facilitate the identification of particular phenotypic profiles which allow a more comprehensive characterization of leukemic cells.[18,24] Thus, we have shown that a high proportion of AML patients display *leukemia-associated* phenotypes, which are distinct from normal progenitor myeloid cells.[32] A possible explanation for these phenotypic aberrations displayed by leukemic cells might be the presence of genetic abnormalities that alter the normal pattern of surface antigen expression. In this hypothesis, specific genetic alterations would be reflected by the existence of characteristic phenotypic features. Accordingly, the aberrant expression of different markers on AML blast cells has been associated with specific genetic abnormalities such as inv-16,[33-36] t(8;21)[37,38] and t(15;17)[39,40] among others.[24,41,42] Based on these findings, we explored whether a particular immunophenotype could be specific and sensitive enough to be of utility in the initial screening of cases with PML-RARα gene rearrangements in order to have a rapid tool, complementary to morphology, for diagnosis of APL which would increase the accuracy of the treatment decision-making process. For this purpose we used a large panel of monoclonal antibodies in double and triple-staining combinations and a similar approach to that currently used for the investigation of phenotypic aberrations to detect minimal residual disease in AML patients.[32] Accordingly, the immunophenotypic characterization of AML was performed after the specific identification of leukemic blast cells. In addition, for each antigen, information was obtained not only on its presence/absence but also on its pattern of expression (i.e. fluorescence intensity, homogeneity/heterogeneity). Using this strategy we

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found that APL cases with an M_3 morphology and PML-RARα translocation displayed characteristic features such as a homogeneous expression of CD33-PE and a heterogeneous reactivity for CD13-PE (both markers being positive in all leukemic cells) together with a characteristic differentiation pattern for the CD34-PE/CD15-FITC antigens. In additon, most cases were HLA-DR negative, as previously described,[13-16] and displayed a single major blast cell subpopulation for all the antigens explored except CD34. Multivariant analysis showed that the pattern of CD34/CD15 and CD13 expression together with the number of major blast cell subsets was the best combination of variables for distinguishing between M_3/PML-RARα$^+$ and non-M_3/PML-RARα$^-$ AML cases. Indeed, the sensitivity of immunophenotyping for selecting PML-RARα$^+$ cases was 100% and the specificity 99%.

The subsequent step was to explore the immunophenotype of the AML cases in which discrepant results were produced by morphology and molecular biology techniques. Our results showed that in 3 out of the 4 M3/PML-RARα$^-$ negative cases immunophenotyping supported the molecular findings, while in the fourth patient the immunophenotype was in agreement with an M_3 morphology but not with the absence of PML-RARα. FISH studies supported the immunophenotypic findings in all these 4 discrepant cases since the presence of PML/RARα was confirmed by FISH analysis on metaphase and interphase cells in only the fourth case mentioned above. Moreover, although confirmatory PCR was repeatedly negative in this case, Southern-blot analysis showed the existence of a RARα gene rearrangement at the DNA level. Among the 5 non-M_3 cases which were initially PML-RARα$^+$ immunophenotyping supported the molecular diagnosis in two patients, who at the same time, were positive for the t(15;17) by FISH. By contrast, immunophenotyping supported the morphologic results in the remaining three cases none of which showed the t(15;17) by FISH. After repeating molecular biology studies, two of these last three cases were shown to be PML-RARα negative thus being considered as false positive cases at the initial PCR, presumably due to sample contamination. In fact, as mentioned above, FISH studies confirmed the absence of t(15;17) in these two patients. Thus, a discrepancy remained between PCR(+) and morphology (M_0), FISH(–) and immunophenotype (score 2) in one case (case 21303). Further studies are necessary in order to elucidate the reasons underlying such discrepancies.

In summary, our results show that the combined use of three phenotypic criteria (presence of a single major blast cell population, heterogeneous reactivity for CD13 and the pattern of expression of CD34/CD15) has great sensitivity (100%) and specificity (99%) for the screenning of those AML cases in which molecular investigation of the PML-RARα gene rearrangements should be performed. Further prospective studies in larger series of AML patients are necessary to confirm our observations.

Contributions and Acknowledgments

AO designed the study, interpreted the final results and wrote the article. MCC carried out the RT-PCRs of all samples. AMB and MCL-B analyzed and reviewed all immunophenotypic studies. RG-S interpreted all PCRs and performed the statistical analysis. He and MG were responsible for the PCR laboratory and clinical management, and contributed in the writing of the paper and literature review. MAG-M was responsible for the morphologic review. MDT and AIR carried out the FISH analyses, together with JH-R, who was, in addition, responsible for the conventional cytogenetics. JFSM was the main coordinator of the group and he reviewed the article to obtain the final form in which it was sent for submission. The order tries to take into account the time, work and scientific contribution of all authors.

Funding

This work was partially supported by a grant from "Fondo de Investigaciones Sanitarias del Ministerio de Sanidad y Consumo" number 98/1156, Madrid, Spain. A. Bortoluci is a recipient of a Grant from the "Instituto de Cooperación Iberoamericana", Madrid, Spain.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received November 13, 1998; accepted January 20, 1999.

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Haematologica 1999; 84:413-418 original paper

Quantitative analysis of CD79b, CD5 and CD19 in mature B-cell lymphoproliferative disorders

ELENA CABEZUDO, PAULA CARRARA, RICARDO MORILLA, ESTELLA MATUTES
Academic Department of Haematology and Cytogenetics, The Royal Marsden Hospital and Institute of Cancer Research, London, UK

ABSTRACT

Background and Objective. **Distinction between B-cell chronic leukemias can be difficult due to overlap in cell morphology and immunologic features. We investigated, by quantitative flow cytometry, the expression of CD79b, CD5 and CD19 in cells from a variety of B-cell disorders to see whether this analysis adds further information useful to the diagnosis and characterization of these diseases.**

Design and Methods. **Peripheral blood cells from 6 normal individuals were used as reference controls. The diseases of the 63 patients investigated comprised: 29 chronic lymphocytic leukemia (CLL), six of them with atypical morphology, 6 B-cell prolymphocytic leukemia (PLL), 12 splenic lymphoma with villous lymphocytes (SLVL) and 16 mantle-cell (Mc) lymphoma in leukemic phase. The study was carried out by triple immunostaining with directly conjugated monoclonal antibodies (MoAb) against CD79b, CD5 and CD19 and quantitative estimation of the antigens per cell assessed with standard microbeads (Quantum Simply Cellular).**

Results. **Compared to normal B-cells, the number of CD19 molecules was significantly lower in cells from all of the B-cell disorders except PLL. The intensity of CD5 in leukemic B-cells was significantly higher in CLL cells, including atypical cases, and Mc lymphoma than in normal B-cells, whilst PLL and SLVL had values similar to those of normal B-lymphocytes. CD79b was expressed at lower levels in all types of leukemic cells compared to normal B-lymphocytes but differences were statistically significant in CLL, Mc lymphoma and SLVL. The number of CD79b molecules per cell was significantly lower in typical CLL than in the remaining B-cell diseases whilst the comparison of CD5 and CD19 intensity between CLL and non-CLL samples failed to show any statistically significant difference.**

Interpretation and Conclusions. **Distinct antigen density patterns for the various conditions emerged from this analysis: Typical CLL was characterized by moderate CD5 and weak or negative CD79b expression. Mc lymphoma showed an homogeneous pattern, characterized by similar expression of CD5 than CLL but significantly stronger expression of CD79b whilst PLL and SLVL had weak CD5 and moderate CD79b expression. Atypical CLL had an intermediate pattern of CD79b antigen expression ranging from weak to moderate with bright CD5. Unlike CD5 and CD79b, CD19 did not discriminate the various B-cell disorders but only between normal and leukemic cells.**
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Correspondence: Estella Matutes, MD, PhD, Academic Department of Haematology and Cytogenetics, The Royal Marsden Hospital, Fulham Road, London SW3 6JJ, UK.
Phone: international +44-171-3528171 ext: 2876 – Fax: international +44-171-3516420 – E-mail: estella@icr.ac.uk

Key words: lymphoma, quantification, flow cytometry, CD79b

The characterization of the B-cell chronic lymphoproliferative disorders is based on a constellation of clinical and laboratory features. Immunophenotyping has contributed to improving the diagnosis of these disorders.[1,2] There is, however, some overlap in marker expression among these entities that makes their classification difficult even when all available diagnostic tools are used. A proportion of chronic lymphocytic leukemia (CLL) cases have atypical morphologic features with the presence of larger cells, eg prolymphocytes or immunoblasts, and/or cleaved or lymphoplasmacytic cells.[3] These cases present problems of differential diagnosis with other B-cell disorders arising with a leukemic phase, particularly mantle cell (Mc) lymphoma or B-cell prolymphocytic leukemia (PLL) and splenic lymphoma with villous lymphocytes (SLVL).[4-6]

By exploiting differences in the intensity of antigen expression quantitative flow cytometry has provided further useful information for the characterization and precise diagnosis of lymphoid disorders as well as for the detection of minimal residual disease.[7-10] Various combinations of markers have been applied in order to distinguish B-cell chronic disorders from normal B-cells. Although CLL can be distinguished from the other B-cell diseases by its distinct immunologic profile,[1] there is, so far, no individual marker or combination of them characteristic for any of the other mature B-cell diseases, e.g. Mc lymphoma, PLL or SLVL.

CD79b (CB3-1) identifies an extracellular epitope of the IgGb/B29 component of the B-cell receptor.[11] Two recent studies that have analyzed the expression of SN8 (assigned to the same cluster designation) on cells from patients with B-cell disorders, have demonstrated the role of this antibody as a useful marker

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for distinguishing CLL from non-CLL cases.[12,13] The discriminatory power of CD79b in other B-cell conditions has not been documented.

In this study we investigated, by quantitative flow cytometry, the expression of CD79b, CD5 and CD19 on cells from a variety of B-cell disorders evolving with a leukemic picture to see whether differences in the intensity of expression of these antigens may disclose patterns which help in the differential diagnosis of the various entities. A PE conjugated CD79b antibody was used as this fluorochrome has a higher sensitivity to detect weakly expressed antigens as is the case of CD79b in CLL.

Design and Methods

Samples

The study was performed at The Royal Marsden Hospital on cryopreserved peripheral blood mononuclear cells from 63 patients with a chronic B-cell lymphoproliferative disorder. These comprised: CLL (29 cases) including 6 cases with atypical morphology (atypical CLL), PLL (6 cases), SLVL (12 cases) and Mc lymphoma in leukemic phase (16 cases). Six samples of cryopreserved peripheral blood mononuclear cells from healthy donors were used to establish reference normal values for the antigen expression of CD79b, CD5, and CD19.

The diagnosis of the various lymphoid diseases was based on cell morphology and immunophenotyping, and when relevant, histology and cytogenetics or fluorescence *in situ* hybridization (FISH) analysis. Typical CLL had a predominant lymphoid population with circulating cells having nuclear clumped chromatin and scanty cytoplasm and less than 10% larger cells whilst atypical CLL had more than 10% prolymphocytes or immunoblasts (CLL/PL) or over 15% cleaved or lymphoplasmacytic cells.[3] PLL was defined by the presence of more than 55% of cells with a prominent central nucleolus. Circulating cells in Mc lymphoma were medium-sized with dispersed speckled chromatin and scanty pale cytoplasm, the majority with an indented nucleus; cells from all the latter cases had the t(11;14) demonstrated either by conventional cytogenetics and/or FISH.[14] Lymphocytes in SLVL had condensed chromatin and short and thin cytoplasmic villi. Spleen histology confirmed the diagnosis of SLVL in 8 of the 12 cases in which this was available.

Immunophenotyping showed that all CLL cases had a typical CLL phenotype according to the previous reported scoring system[1] (CD5, CD23, FMC7, CD22 and surface immunoglobulin) with scores ranging from 3 to 5 while the remaining diseases had phenotypes atypical of CLL with low scores (0-2).

Immunostaining

Mononuclear cells were obtained from peripheral blood samples by density gradient centrifugation with Lymphoprep (Nycomed, Oslo, Norway). After separation and washing the cells were cryopreserved in 10% dimethylsulfoxide and 4% albumin in phosphate buffered saline (PBS) (final concentrations) and stored in liquid nitrogen. Prior to their use, the cells were thawed at 37ºC in a water bath and washed in RPMI 1640 with 10% fetal calf serum. After washing, the cells were resuspended in PBS/azide to a final concentration of 10×10^6 cells per mL. Cell viability, assessed by trypan blue exclusion, was always over 80%. Studies comparing fresh and cryopreserved cells were not carried out, but we previously reported that there are no differences in the antigen density of CD5 and CD19 between cryopreserved and fresh cells.[15] Because studies in all cases here were performed on cyopreserved cells, results were comparable. The analysis was performed using a triple staining combination with directly labeled MoAb: CD5-FITC (DK23) (Dako), CD79b-PE (CB3-1) (Immunotech S.A., Marseille, France) and CD19-TC (SJ25-C1) (Caltag Laboratories, San Francisco, CA, USA). Mouse immunoglobulins of IgG1 subclass FITC, PE and TC labeled were used as negative controls in every experiment. To each tube 50 mL of 2% AB serum were added to the cells, 1×10^6 per test, followed by the three MoAb mixture and incubated for 15 minutes at room temperature. After incubation, the cells were washed with phosphate buffered solution (PBS) azide, the supernatant discarded and resuspended in 0.5 mL Isoton (Coulter, Hialeah, FL, USA).

Assessment of antibody binding capacity by Quantum Simply Cellular Method

Quantitative analysis was performed as described elsewhere.[15] Briefly, Quantum Simply Cellular Microbeads Kit (Sigma, St Louis, Mo., USA.) provides a method for the evaluation of the number of molecules of antibody per cell by flow cytometry.[16] This kit has a mixture of four types of microbeads coated with different amounts of goat anti-mouse immunoglobulin with a precalibrated antibody binding capacity (ABC). The microbeads react with directly labeled mouse MoAb and serve as a set of standards to calibrate the fluorescence scale of the flow cytometer for each antibody, thus converting the MIF into the number of molecules of antigen expressed per cell. Microbeads were incubated separately with each MoAb and the acquisition was also carried out separately for each reagent using the same procedure as for the clinical samples. For each sample, the ABC value of the isotypic control was subtracted from the ABC value of the positive cells. The microbeads, 50 µL per tube, were incubated with each MoAb separately, washed and further processed as the clinical samples and finally acquired on the flow cytometer under the same conditions.

Flow cytometry analysis

Data acquisition was performed on a FASCcan flow cytometer (Becton Dickinson) using the LYSYS II

software (Becton Dickinson). For data analysis, Paint-A-Gate software (Becton Dickinson) was used. Peripheral blood mononuclear cells labeled with CD4/CD8/CD3 were used to set the calibration of the instrument and fluorescence compensation. For each tube 10^4 cells or microbeads were acquired.

Statistical analysis

Results were expressed as median, standard deviation (s.d.) and compared with the Mann-Whitney U test. A two-sided p value ≤ 0.05 was considered statistically significant. Median values were chosen due to the dispersion of values in some diseases, such as B-PLL and atypical CLL.

Results

Normal samples

There was little variability in the expression of the three B-cell associated antigens analyzed in normal samples (Figures 1-3). As shown in Table 1, the median number of CD19 molecules per cell (ABC values) expressed in normal peripheral blood B lymphocytes, was $13\pm1\times10^3$/cell (range $11\text{-}16\times10^3$); $11\pm2\times10^3$/cell (range $8\text{-}13\times10^3$) for CD5 and $43\pm8\times10^3$/cell (range $29\text{-}55\times10^3$) for CD79b.

Leukemic samples (Table 1).

CD19 antigen. CD19 was consistently expressed in cells from all B-cell leukemias. Compared to normal controls, the intensity of antigen expression was significantly lower in all the conditions except in PLL. Over 80% of leukemic samples had less than 10×10^3 CD19 molecules per cell and thus, below the lowest value of normal B cells (Figure 1). Cells from very few cases had values within the normal range. There were no statistically significant differences regarding the expression of this marker between CLL and non-CLL cases or among the other groups of B-cell malignancies, although the highest values were found in PLL.

CD5 antigen. CD5 was positive in typical CLL and Mc lymphoma cases, 83% of atypical CLL, 83% of PLL and in 50% of SLVL cases. The intensity of CD5 expression in leukemic B-cells was significantly higher in CLL, including atypical cases, and Mc lymphoma than in normal B-cells (Figure 2). Analyzing the CD5 positive samples, the comparison of CD5 intensity of expression between CLL and non-CLL samples or among other non-CLL B-cell disorders failed to show any statistically significant difference.

Table 1. Antibody binding capacity values ($\times10^3$) in normal and leukemic B-cells (median ± standard deviation)#.

Antigen	*Normal n=6*	*CLL n=23*	*a-CLL n=6*	*B-PLL n=6*	*Mc-lymph. n=16*	*SLVL n=12*
CD19	13±1	4±4	5±2	8±7	4±4	4±5
p value*		p=0.001	p=0.003	NS	p=0.002	p=0.006
p value°			NS	NS	NS	NS
CD5	11±2	31±17	40±23	16±10	24±14	18±9
p value*		p=0.002	p=0.01	NS	p=0.009	NS
p value°			NS	NS^	NS	NS
CD79b	43±8	2±3	6±31	37±18	17±16	12±13
p value*		p<0.001	NS	NS	p=0.009	p=0.003
p value°			p=0.02	p=0.001	p=0.001	p=0.001

Abbreviations: CLL = chronic lymphocytic leukemia; a-CLL= atypical CLL; PLL= B-cell prolymphocytic leukemia; Mc-lymphoma = mantle cell lymphoma; SLVL= splenic lymphoma with villous lymphocytes.
#Only the positive cases are included in the statistical analyses.
p value: comparison between normal B-cells and leukemic cells.*
p value°: comparison between CLL and other leukemia cells.
^Differences on CD5 molecules were significant between PLL plus SLVL versus CLL (p=0.01).

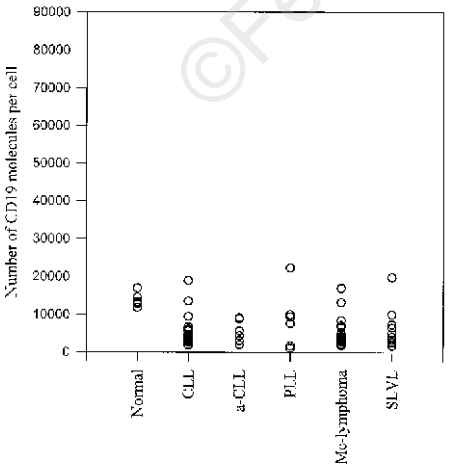

Figure 1. Distribution of antibody binding capacity (ABC) values for CD19 in normal controls, CLL, atypical CLL, PLL, mantle cell lymphoma and SLVL cases. Each circle represents a different sample.

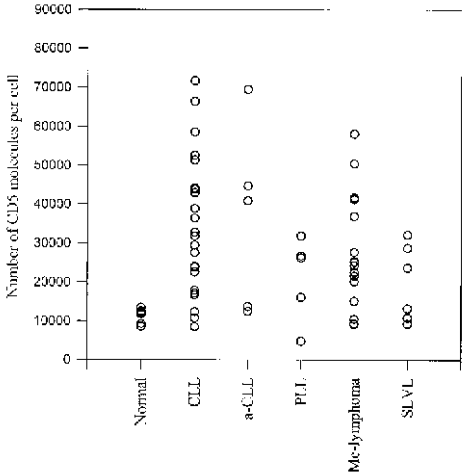

Figure 2. Distribution of antibody binding capacity (ABC) values for CD5 in normal controls, CLL, atypical CLL, PLL, mantle cell lymphoma and SLVL cases. Each circle represents a different sample.

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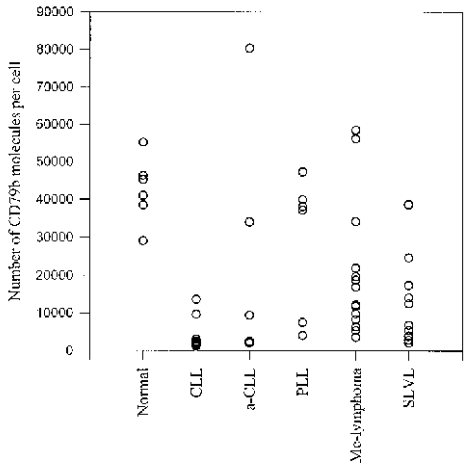

Figure 3. Distribution of antibody binding capacity values for CD79b in normal controls, CLL, atypical CLL, PLL, mantle cell lymphoma and SLVL cases. Each circle represents a different sample.

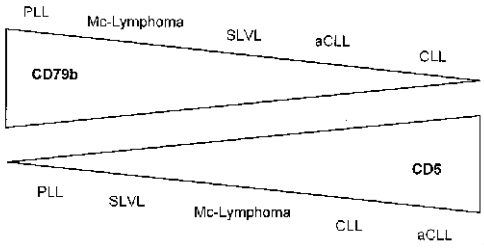

Figure 4. Diagram illustrating the intensity of CD79b and CD5 in B-cell disorders.

However, when we compared the group of PLL plus SLVL versus CLL the differences were significant with lower CD5 expression in PLL and SLVL.

CD79b antigen. CD79b was negative in 6 of the 23 CLL cases and consistently positive in the remaining B-cell disorders. Cells from all leukemia and lymphoma patients had lower CD79b expression than normal B-cells, although results were only significantly lower for typical CLL, Mc lymphoma and SLVL. Among the positive cases, CD79b was weakly expressed (ABC<2×10^3) in cells from 88 % of CLL samples, 50% of atypical CLL and 17% of SLVL. The number of CD79b molecules per cell was higher than 2×10^3 in all Mc lymphoma and PLL cases. Statistical analysis revealed that CD79b expression was significantly lower in typical CLL compared to other B-cell disorders whilst there were no differences among the B-cell leukemias other than CLL albeit there being a trend for PLL cases to have higher CD79b expression. There was also significantly higher CD79b expression in atypical CLL than typical CLL, albeit lower than in the other diseases (Figure 3).

According to our findings, the combination of CD5 and CD79b emerged as a useful marker to distinguish CLL from non-CLL cases and, to some extent, among some of the other B-cell disorders. Figure 4 tries to illustrate the pattern of antigen expression. Typical CLL was characterized by moderate expression of CD5 and weak or negative expression of CD79b. Mc lymphoma showed a homogeneous pattern, characterized by a similar intensity of expression of CD5 as that found in CLL but significantly stronger expression of CD79b (Figure 5) whilst SLVL and PLL had weaker CD5 expression and stronger CD79b than that in CLL.

Discussion

We investigated the value of quantitative flow cytometry, analyzing the intensity of expression of three antigens: CD19, CD5 and CD79b to see whether this analysis can be of diagnostic value for differentiating CLL from non-CLL cases and also whether it can distinguish among the B-cell disorders other than CLL.

We have shown that although CD19 discriminates well between normal and leukemic B-cells, it does not help to distinguish among the various conditions. In contrast, the antigen density of CD5 combined with that of CD79b is not only useful for differentiating normal from leukemic B-cells but also helpful in distinguishing among some of the various B-cell disorders. Thus, although Mc lymphoma and CLL had a similar CD5 antigen density, the number of CD79b molecules in Mc lymphoma was significantly higher than in CLL. Of interest was the intermediate pattern of CD79b antigen expression in atypical

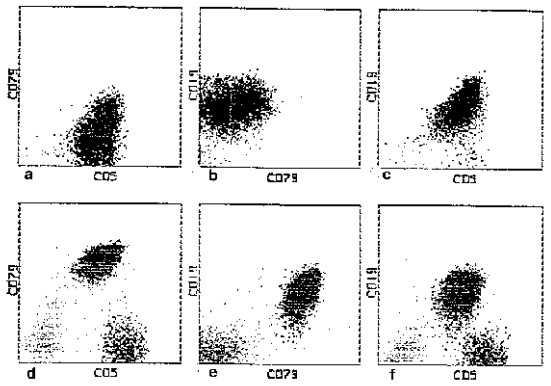

Figure 5. Triple labeling with CD5/CD79b/CD19 (FITC/PE/TC) in peripheral blood lymphocytes:
a, b, c: Dot-plots showing the clonal population in a case of CLL. The weak expression of CD79b contrasts with the moderate expression of CD19 and the strong positivity of CD5, similar to the residual T lymphocytes.
d, e, f: Dot-plots showing the clonal population in a case of mantle cell lymphoma with a high expression of CD79b, and CD5 and CD19 expression comparable to the CLL case.

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CLL, ranging from weak to moderate and lying between that in CLL and other B-cell disorders and indeed being significantly higher than in typical CLL.

The transmembrane forms of all immunoglobulin (Ig) classes are associated with two glycoproteins, mb1 and B29 that are essential for signal transduction following antigen binding to the Ig molecule.[17-19] CD79b (CB3-1) recognizes an external epitope of the B29 component of the surface immunoglobulin receptor complex (B cell receptor).[11,20] The B29 protein is expressed early in the cytoplasm in B-cell precursors albeit later than mb-1 (CD79a), CD19 or cytoplasmic CD22, whereas surface expression occurs concomitantly with surface immunoglobulin expression in all B-cells.[21-22] A recent study has documented that CLL cells have decreased surface expression of B29 (CD79b) and in some cases, undetectable B29 m-RNA.[23] Further, most of those cases with detectable m-RNA had point mutations or deletions in the cDNA located in the B29 transmembrane and cytoplasmic domains, probably underlying the loss of signal transduction of CLL cells.[23,24] The low number of CD79b molecules per cell exhibited by CLL cells correlates well with low surface inmunoglobulin and CD22 expression characteristic of this disease.[1,25] Although previous studies have documented that the majority of CLL cases do not stain with CD79b (SN8),[12,13] the use of a MoAb directed against a different epitope (CB3-1) and a brighter (PE) and directly labeled antibody allows the detection of small numbers of CD79b molecules in CLL lymphocytes. Indeed, this may correlate with the documented molecular findings of CD79b in CLL.

From the diagnostic point of view, the quantitative estimation of CD79b together with that of CD5 seems to be discriminative between CLL and non-CLL cases whilst the antigen density of CD19 is a good discriminative marker between leukemic and normal B-cells. When considering the antigen density of CD5 and CD79b in the remaining B cell disorders, Mc lymphoma exhibited the most homogeneous pattern, clearly different from that in both typical and atypical cases of CLL. There was also a trend for a different pattern of CD5 and CD79b antigen expression in the other two B-cell disorders, PLL and SLVL, with lower numbers of CD5 than in CLL and Mc-lymphoma and moderate to bright CD79b. Problems of differential diagnosis usually arise between Mc lymphoma and atypical CLL, PLL and SLVL and quantitative flow cytometry might be an additional tool for distinguishing between them. Analysis of greater numbers of non-CLL cases e.g. PLL, SLVL and atypical CLL is needed to substantiate these findings.

In addition to the diagnostic value, the differences in antigen expression of CD19, CD5 and CD79b between normal and leukemic samples has potential for monitoring treatment and can be exploited to detect minimal residual disease.

Contributions and Acknowledgments

EC, RM and EM were involved on the immunophenotyping data and interpretation of the results. Morphology review was performed by EM. PC carried out the FISH analysis for the 11;14 translocation.

Funding

EC was supported by a grant from the Spanish Government (FIS nº 96/5035; 96/5509).

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received November 12, 1998; accepted January 28, 1999.

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Haematologica 1999; 84:419-424 original paper

Expression of thrombospondin receptor (CD36) in B-cell chronic lymphocytic leukemia as an indicator of tumor cell dissemination

SERGIO RUTELLA,*° CARLO RUMI,*° PIERLUIGI PUGGIONI,*° TIZIANO BARBERI,*° ANTONELLA DI MARIO,* LUIGI MARIA LAROCCA,^ GIUSEPPE LEONE*

**Chair of Hematology, °Center for the Flow Cytometric Study of Blood Cells and ^Department of Pathology, Catholic University, Rome, Italy*

ABSTRACT

Background and Objective. **The expression of CD36 antigen has not been conclusively associated with human B-lymphocytes although CD36 was recently detected in a human B-cell angiotropic lymphoma where it might be involved in lymphoblast-endothelial cell adhesion. We investigated the expression of CD36 in B-cell chronic lymphocytic leukemia (CLL) by multiparameter flow cytometry; results were correlated with clinical features.**

Design and Methods. **CD36 expression was evaluated on peripheral blood and bone marrow samples from 24 patients affected by $CD5^+$ B-CLL. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation, were labeled with fluorochrome-conjugated monoclonal antibodies under standard experimental conditions and were analyzed by flow cytometry. CD36 expression was quantified both in terms of frequency of $CD19^+CD36^+$ cells and of mean fluorescence intensity (MFI-R) of $CD36^+$ cell populations. The intensity of CD36 expression was arbitrarily classified as weak (MFI-R ranging from 3 to 6; score 0), moderate (MFI-R ranging from 6 to 9; score 1), intermediate (MFI-R ranging from 9 to 11; score 2) or strong (MFI-R ranging from 11 to 17; score 3).**

Results. **CD36 could be detected on 3% (range 2-5) of normal $CD19^+$ B-lymphocytes and on 45% (range 30-75) of neoplastic $CD19^+$ B-cells. When CLL patients were stratified according to CD36 staining intensity, higher hemoglobin levels (Hb) were recorded in patients assigned to score 0 (Hb = 14.3 g/dL; range 13.9-15.1) compared to patients scoring 1-2 (Hb = 11.2; range 10.3-12.2) or 3 (Hb = 9.8; range 9.6-11.6; p=0.0053). Similarly, higher platelet counts (Plt) were found in patients scoring 0 (Plt = 282×10^3/μL; range 244-319), compared to patients with intermediate (Plt = 175×10^3/μL; range 144-238) and high scores (Plt = 149×10^3/μL; range 103-230; p=0.044); lymphocyte count (Ly) was significantly higher in patients assigned to score 3-4 (Ly = 23.3×10^3/μL, range 13-30) compared to score 0-2 (Ly = 9.8×10^3/μL, range 8.5-10.8; p=0.045). CLL patients expressing CD36 at intermediate-to-strong intensity (MFI-R = 14, range 9-16) were more frequently assigned to Rai stages III-IV than stages I-II (CD36 MFI-R = 9, range 6.5-11; p=0.005) and stage 0 (CD36 MFI-R = 6, range 4-7.3; p<0.001). Interestingly, bone marrow diffuse histology was strongly associated with higher CD36 expression (MFI-R = 8.7; range 4.7-13.9) compared to non-diffuse patterns of bone marrow infiltration (MFI-R = 6.7; range 5.2-9.3; p=0.0019). In multivariate regression analysis, CD36 staining intensity significantly and independently correlated with diffuse BM histology (p=0.033).**

Interpretation and Conclusions. **The present report provides the first evidence of CD36 expression on $CD19^+$ B-cells from CLL; the correlations with clinical parameters strongly support the view that CD36 might favor tumor cell spreading. Whether high CD36 expression levels on CLL $CD19^+$ B-cells identify an aggressive disease subset remains to be further confirmed in larger series of patients.**
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Key words: CD36, chronic lymphocytic leukemia, flow cytometry, tumor spreading

Correspondence: Sergio Rutella, MD, Department of Hematology, Center for the Flow Cytometric Study of Blood Cells, Catholic University, largo A. Gemelli 8, 00168 Rome, Italy. Phone: international +39-06-30154968 – Fax: international +39-06-3312538 – E-mail: srutell@tin.it

CD36 is a membrane-bound glycoprotein receptor for thrombospondin (TSP), identical to platelet GPIV; CD36 is expressed on a variety of hematopoietic cell types and cultured cell lines and mediates several TSP-dependent reactions, namely inflammation, chemotaxis, atherosclerosis, cell proliferation and tumor metastasis.[1,2]

Among hematopoietic cells, monocytes, megakaryocytes and erythroid precursors express CD36; in particular, CD36 regulates the adhesion of erythroid progenitors to marrow matrix fibroblasts and its expression declines during erythroid differentiation, while blasts from megakaryocytic leukemias have been shown to up-regulate CD36 following culture in the presence of lymphocyte conditioned medium and interleukin-3.[3] Interestingly, the expression of CD36 is restricted to vessels in tonsils and lymph nodes and has been detected by immunohistochemistry in scattered cells of thymic medulla and cortex.[4] Furthermore, CD36 mediates adhesion of human erythrocytes infected by *P. falciparum* to cap-

illary endothelial cells, thus contributing to morbidity and mortality of human malaria.[1]

An association of CD36 with tyrosine kinases of the src gene family has been described in platelets and endothelial cells, thus extending the role of CD36 to that of a signal transduction molecule;[1] interestingly, an intracellular localization of CD36 has been demonstrated in human uninduced monocytic cell lines and in platelets, where CD36 is stored in α-granules and is rapidly transported to the plasma membrane upon activation.[5]

The expression of CD36 has not been conclusively associated with human B lymphocytes although CD36 was recently detected in a human B-cell angiotropic lymphoma, where it might be involved in lymphoblast-endothelial cell adhesion;[6] in contrast, primary murine B cells and cell lines have been shown to express CD36.[7] In the present investigation, B-cells from chronic lymphocytic leukemia (CLL) at diagnosis were analyzed by multiparameter flow cytometry for CD36 expression. Given the potential role of CD36 in mediating adhesion to endothelial cells and in promoting tumor cell spreading, the results were correlated with clinical features, namely peripheral blood lymphocytosis, platelet count and hemoglobin level, presence of splenomegaly and/or extranodal involvement, pattern of bone marrow infiltration and staging according to Rai.[8,9]

Design and Methods

Patients and samples

Twenty-four consecutive patients [median age 64 years (range 55-70)] referred to our Institution from April 1997 and affected by $CD5^+$ B-cell chronic lymphocytic leukemia (B-CLL) were evaluated at diagnosis, except for 4 patients who, at the time of inclusion in the present study, were in disease remission after conventional chemotherapy; the patients' characteristics are summarized in Table 1.

B-CLL was diagnosed according to accepted morphologic and immunophenotypic criteria, including analysis of κ or λ immunoglobulin light chain restriction.[10,11] Patients were clinically evaluated to assess liver, spleen or lymph node enlargement. Total leukocyte, lymphocyte (Ly) and platelet (Plt) counts as well as hemoglobin (Hb) level were determined by means of automated hematologic analyzers. The pattern of bone marrow infiltration was classified as diffuse or non-diffuse (nodular, interstitial or mixed nodular-interstitial), as previously reported.[8]

The expression of CD36 antigen was also investigated on normal peripheral blood samples obtained from ten healthy donors after their informed consent. The present study was approved by the Institutional Human Research Committee.

Immunologic markers

Mononuclear cells (MNCs) were isolated from heparinized peripheral blood (PB) and/or bone marrow (BM) as follows: samples were diluted 1:2 in RPMI 1640 complete culture medium, layered onto Ficoll-Hypaque gradient (density=1,077 g/L, Uppsala, Sweden) and centrifuged at 1700 rpm for 30 minutes at 20°C. Cells at the interface were harvested, washed in RPMI 1640 at 1500 rpm for 6 minutes and suspended at a final concentration of 1×10^6/mL. In 15 out of 24 CLL patients, BM samples were available and could be used for flow cytometry analysis.

Cytophilic immunoglobulins were shed by preincubation at 37° for 30 minutes.[12] For surface membrane labeling, PB or BM MNCs were incubated for 30 minutes at 4°C with pre-titrated dilutions of the following fluorescein isothyocyanate (FITC)-, phycoerythrin (PE)- or peridinin chlorophyll protein (PerCP)-conjugated monoclonal antibodies (mAb): CD5 (L17F12 clone, IgG_{2a}), CD3 (SK7 clone, IgG_1), CD19 (4G7 clone, IgG_1), CD20 (L27 clone, IgG_1), CD22 (S-HCL-1 clone, IgG_{2b}), CD23 (EBVCS-5 clone, IgG_1), HLA-DR (L234 clone, Ig_{2a}), CD4 (SK3 clone, IgG_1), CD8 (SK1 clone, IgG_1), CD16 (B73.1 clone, IgG_1), CD11c (S-HCL-3 clone, IgG_{2b}), CD25 (2A3 clone, IgG_1; all from Becton Dickinson [BD], CA), CD138 (B-B4 clone, IgG_1; Valter Occhiena, Turin, Italy), anti-κ/anti-λ immunoglobulin light chain (IgG_1; BIO D, Bari, Italy) or fluorochrome-conjugated isotype-matched irrelevant mAb to establish background fluorescence.[13] After labeling, cells were thoroughly washed with phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) and were kept on ice until flow cytometry analysis.

Detection of CD36 antigen

Aliquots of PE- and/or PerCP-surface labeled normal and neoplastic MNCs were incubated for 30 minutes at 4°C with pre-titrated dilutions of uncon-

Table 1. Characteristics of CLL patients (n = 24).

Age, median (range)	70.5 (65-75)
Male:female ratio	14/10
WBC, median (range)	15.8 (11.3-21.3)
Ly, median (range)	10.1 (5.8-13.9)
Hb, median (range)	12.7 (11.4-13.9)
Plt, median (range)	210 (129-263)
Stage	
0	(6/24)*
I-II	(8/24)
III-IV	(6/24)
CR	(4/24)
Spl	4/16 (25%)
Extranodal	5/19 (26.3%)

WBC = white blood cells; Ly = lymphocytes; Hb = hemoglobin; Plt = platelets; Spl = splenomegaly; Extranodal = extranodal disease. Data were expressed as median and interquartile range. All patients were analyzed at diagnosis, except for 4 patients who, at the time of inclusion in the present study, were in disease remission (CR) after conventional chemotherapy.

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jugated anti-CD36 mAb (SMO clone, IgM, BIO D) or with irrelevant isotype-matched mAb as control; after washings in PBS supplemented with EDTA 0.1 mM, cells were incubated for 30 min at 4°C with saturating amounts of FITC-conjugated goat-anti-mouse mAb (Ortho Diagnostics Syst., Raritan, NJ, USA). After incubation, cells were washed in PBS-1%BSA and were kept on ice until flow cytometry analysis. Platelet-rich plasma (PRP) obtained after centrifugation of normal PB samples at 800 rpm for 10 minutes was used as the positive control for CD36 staining.

Flow cytometry

All samples were run through a FACScan flow cytometer (BD), equipped with an argon laser emitting at 488nm. Forward (FSC) and side scatter (SSC) were collected as linear signals and all fluorescent emissions were collected on a four-decade logarithmic scale. FITC, PE and PerCP signals were measured at 530 nm, 575 nm and 670 nm, respectively, and spectral overlap was minimized by electronic compensation with Calibrite Beads (BD) before each determination series. A minimum of 10,000 events was acquired in list mode using CellQuest® software (BD). The percentage of $CD19^+$ leukemic cells co-expressing a given antigen (Ag) was calculated according to the formula:

$$\%\ Ag^+ = \frac{\text{Nr of } CD19^+Ag^+}{\text{Nr of } CD19^+Ag^- + \text{Nr of } CD19^+Ag^+} \times 100$$

The staining intensity was calculated as the mean fluorescence intensity ratio (MFI of test histogram : MFI of control histogram), as previously reported.[14]

Immunofluorescence analysis

MNCs were smeared on glass slides by cytospin centrifugation and were fixed for 5 minutes at room temperature with absolute methanol and for 2 minutes at –20°C with acetone.[15] After rehydration in PBS, cells were incubated for 30 minutes at 37°C with a proper dilution of unconjugated anti-CD36 mAb. After extensive washing in PBS, cells were incubated for 30 minutes at room temperature with a 1:20 dilution of FITC-conjugated goat-anti-mouse mAb (Ortho Diagnostics Syst.). The slides were then washed in PBS, mounted in PBS/glycerol and observed under an epifluorescence microscope (Axiophot; Zeiss, Jena, Germany), using a x63 objective. Cells were photographed using a 400 ASA black and white film (Tmax; Eastman Kodak Co., Rochester, NY, USA).

Statistical methods

To test the approximation of population distribution to normality, y (g) statistics (for symmetry and kurtosis testing) as well as probability plots were used.[16] Data were asymmetrically distributed and were presented as median and interquartile range (25^{th} and 75^{th} percentile). Consequently, statistical analyses were performed using the Mann Whitney U test or the Kruskal-Wallis test for unpaired determinations and the χ^2-test, as appropriate. The correlations between series of data were calculated using Spearman rank analysis. Multivariate regression models were formed to examine the relation between the dependent variable (BM histology) and potential predictor variables, including percentage of CD36-expressing B-cells, CD36 staining intensity, PB lymphocytosis, Hb level and Plt count. The criterion for statistical significance was defined as $p<0.05$.

Results

Expression of CD36 on normal peripheral blood cells

CD36 could be detected on 3% (range 2-5) of normal $CD19^+$ B-lymphocytes and on a subset (< 2%) of $CD4^+$ as well as $CD8^+$ peripheral T-lymphocytes; 4% (range 2-5) of natural killer (NK) cells, defined by the co-expression of CD16 and CD56 and by the lack of CD3 antigen, were dimly stained by the anti-CD36 mAb. In accordance with previously published reports,[1] more than 90% of circulating $CD14^+$ monocytes expressed detectable amounts of CD36 (MFI-R = 5, range 3.2-6.5); conversely, no reactivity above background fluorescence could be evidenced on circulating $CD16^+$ granulocytes. As expected, normal platelets were uniformly and intensely stained by the anti-CD36 mAb (MFI = 10, range 7.7-11.5).

Expression of CD36 on CLL B-cells

CD36 antigen could be readily detected on 45% (range 30-75) of neoplastic B-cells from patients with CLL at diagnosis (Figure 1). These findings were fur-

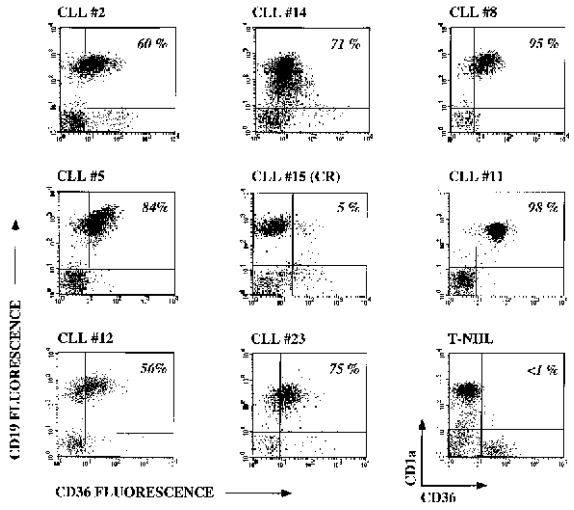

Figure 1. Flow cytometric detection of CD36 in representative B-CLL samples. CR = complete remission. The absence of reactivity with anti-CD36 mAb in a case of CD1a+ T-cell non-Hodgkin lymphoma is shown for comparison.

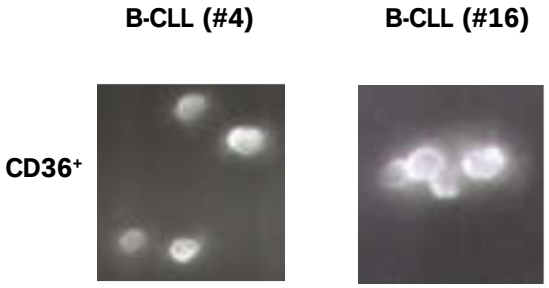

Figure 2. Immunofluorescence analysis of CD36 expression in PB samples from two representative B-CLL cases.

ther confirmed by microscopy analysis (Figure 2). The majority of CLL (62%) expressed CD36 on < 40% of $CD19^+$ B-cells; the remaining CLL cases expressed CD36 on intermediate-to-high percentages (40-80%) of B lymphocytes. No differences were observed when comparing either the frequency of CD36-coexpressing B-cells or CD36 staining intensity in PB and BM samples from individual patients (data not shown).

Since the entire $CD19^+$ $CD36^+$ B-cell population appeared to be shifted by the CD36 staining in bidimensional cytograms, the intensity of CD36 expression was arbitrarily classified as weak (MFI-R ranging from 3 to 6; score 0), moderate (MFI-R ranging from 6 to 9; score 1), intermediate (MFI-R ranging from 9 to 11; score 2) or strong (MFI-R ranging from 11 to 17; score 3).

The majority of CLL (83.3%) expressed CD36 at low-to-intermediate intensity (MFI-R ranging from 3 to 11) and only a minority of CLL (14%) expressed CD36 at intermediate-to-strong intensity (MFI-R ranging from 11 to 17). It was of interest to observe that CLL patients who, at the time of inclusion in the present study, were in disease remission after conventional chemotherapy showed a negligible amount of circulating and BM $CD19^+$ $CD36^+$ B-lymphocytes; a representative flow cytometric profile from one of these cases is shown in Figure 1.

Correlation between CD36 expression and clinical and laboratory features

To ascertain whether CD36 expression level might be associated with a greater propensity to tumor cell spreading, phenotypic features were correlated with laboratory and clinical parameters, namely PB lymphocytosis, Plt count and Hb level, presence of splenomegaly and/or extranodal involvement, pattern of BM infiltration and staging according to Rai.

When CLL patients were stratified according to CD36 staining intensity, higher Hb levels were recorded in patients assigned to score 0 (Hb = 14.3 g/dL; range 13.9-15.1) compared to patients scoring 1-2 (Hb = 11.2; range 10.3-12.2) or 3 (Hb = 9.8; range 9.6-11.6; p=0.0053). Similarly, higher Plt counts were found in patients scoring 0 (Plt = 282×10^3/μL; range 244-319), compared to patients with intermediate (Plt = 175×10^3/μL; range 144-238) and high scores (Plt = 149×10^3/μL; range 103-230; p=0.044). Interestingly, Ly count was significantly higher in patients assigned to score 3-4 (Ly = 23.3×10^3/μL, range 13-30) compared to score 0-2 (Ly = 9.8×10^3/μL, range 8.5-10.8; p=0.045).

CLL expressing CD36 at intermediate-to-strong intensity (MFI-R = 14, range 9-16) were more frequently assigned to Rai stages III-IV than stages I-II (CD36 MFI-R = 9, range 6.5-11; p=0.005) or stage 0 (CD36 MFI-R = 6, range 4-7.3; p<0.001; Figure 3); similarly, CLL patients expressing CD36 on a relevant percentage of $CD19^+$ B-cells (75%, range 28-95) were more frequently assigned to Rai stages III-IV than stages I-II (28% $CD19^+$ $CD36^+$ B-cells, range 16-44; *p*=0.015) or stage 0 (10% $CD19^+CD36^+$ B-cells, range 6-15; *p*=0.0178; Figure 3).

A well-defined trend, although statistically not significant, towards a brighter CD36 staining intensity was observed in CLL patients with splenomegaly (MFI-R=8; range 6.6-11) compared to those without spleen involvement (MFI-R = 6; range 3.4-8.3; *p*=NS). Similarly, the correlation between presence of extranodal disease and level of CD36 expression failed to achieve statistical significance, although a trend towards brighter fluorescence intensities could be evidenced in patients with more advanced disease (data not shown). Of interest, BM diffuse histology was strongly associated with higher CD36 expression (MFI-R =

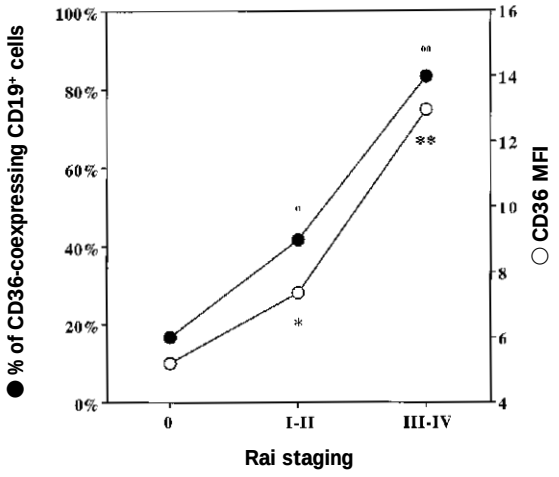

Figure 3. Correlation between the expression of CD36 antigen on $CD19^+$ CLL B-cells both in terms of frequency of $CD19^+CD36^+$ B-cells and in terms of MFI-R and clinical stage according to Rai. °*p*=0.015 and °°*p*=0.0178 compared to stages I-II and stage 0, respectively; **p*=0.005 and ***p*<0.001 compared to stages I-II and stage 0, respectively.

8.7; range 4.7-13.9) compared to non-diffuse patterns of BM infiltration (MFI-R = 6.7; range 5.2-9.3; p=0.0019). When the significance and independence of potential predictor variables with respect to BM histology (i.e. percentage of $CD19^+CD36^+$ leukemic B-cells, CD36 fluorescence intensity, PB lymphocytosis, Hb level and Plt count) were evaluated in multivariate regression analysis, none of those covariables significantly altered the association between diffuse pattern of BM infiltration and brighter CD36 staining intensities (p=0.033).

Discussion

CLL is a clinically and biologically heterogeneous disease, characterized by the clonal proliferation of mature-appearing B-lymphocytes, which represent an antigen-independent pre-follicular state of differentiation.[17] In early stages of disease, CLL B-lymphocytes can be found primarily in PB and BM; however, disease progression is associated with infiltration of lymphoid and non-lymphoid organs, suggesting that microenvironmental factors might change during the disease course, thus affecting tumor cell adhesion and patterns of dissemination.[18]

Although several investigations have specifically focused on the expression and function of cell-adhesion molecules in chronic B-cell leukemias, few reports have correlated phenotypic features with clinical and/or laboratory parameters.[17,19]

Recently, receptors for TSP were identified on invasive metastatic cells of squamous and ductal carcinomas;[20] the staining intensity, as determined in immunohistochemical sections, predicted the development of metastatic disease within 16 months of initial treatment; interestingly, higher antigen densities were associated with a reduced overall survival, suggesting that the TSP-rich extracellular matrix of those neoplasms might favor tumor cell adhesion and migration.

Human CD36 is a membrane-bound receptor for thrombospondin and can be detected on myelomonocytic cells, platelets, megakaryocytes and endothelial cells.[1] Although CD36 has not been apparently associated with human B lymphocytes, the report of a case of CD36-expressing human B-cell angiotropic lymphoma suggests that CD36 might be found at discrete stages of B lymphocyte differentiation.[6] In mice, the CD36 gene has been shown to be a target gene through which the Oct-2 transcription factor can affect B-cell differentiation.[7] As a membrane receptor on B lymphocytes, CD36 might transduce signals which trigger B-cell differentiation in response to intercellular contact or to as yet unrecognized ligand(s). Given its putative function as a receptor for long-chain fatty acids, CD36 might regulate membrane synthesis and provide energy supply during the differentiation to plasma cells. These hypotheses seem further confirmed by the impairment of the antigen-dependent stage of B-cell development and by the reduced tendency to homotypic cell adhesion in Oct-$2^{-/-}$ mice.[7]

In the present report, reactivity with the anti-CD36 mAb was found on a minor subset of normal PB B-lymphocytes; conversely, B-cells from CLL unequivocally expressed CD36 antigen. The specificity of these findings was further confirmed by the lack of reactivity with the anti-CD36 mAb in a case of thymic $CD1a^+$ lymphoma and in a case of pre-B acute lymphoblastic leukemia (personal unpublished observation, 1997).

The majority of CLL was assigned to score 0-2, indicating that only a minority of cases expressed CD36 antigen at high density. When CLL patients were stratified according to CD36 fluorescence intensity, higher Hb levels and Plt counts were found in patients assigned to score 0 compared to those assigned to intermediate and high scores; these findings suggest that brighter CD36 fluorescence intensities might be associated with more advanced or aggressive disease as a result of enhanced adhesive interactions of tumor cells with the BM microenvironment. However, the correlation between CD36 score and splenomegaly or presence of extranodal involvement, although intriguing, failed to reach statistical significance. When CLL patients in disease remission were evaluated, a negligible fraction of $CD19^+CD36^+$ B-cells was detected (< 1%), thus suggesting that the expression of CD36 receptor might be restricted to neoplastic B-lymphocytes.

Recently, patients with diffuse BM infiltration were reported to survive from 2 to 4 years, as opposed to patients with a non-diffuse histology, whose survival ranged between 8 and 10 years.[19] In the present investigation, higher intensities of CD36 staining were recorded in CLL patients with diffuse BM histology, an accepted adverse prognostic factor;[19] moreover, the significant and independent correlation between CD36 staining intensity and BM diffuse histology in multivariate regression analysis conferred to CD36 antigen expression a predictive value on the propensity to neoplastic cell dissemination.

In conclusion, the present report provides the first conclusive evidence of the expression of CD36 antigen on $CD19^+$ neoplastic B-cells. Further investigations are encouraged to analyze the mechanisms of CD36 gene expression in normal and leukemic B-lymphopoiesis; interestingly, low-grade B-cell non-Hodgkin's lymphomas in leukemic phase were recently found to express CD36 antigen on relevant percentages of neoplastic B-cells (manuscript in preparation, 1999). The observation of higher CD36 staining intensities in CLL patients with diffuse BM histology and with reduced Hb level and Plt count strongly suggests that CD36 antigen might mediate tumor cell dissemination and that its expression level might identify patients with more aggressive disease.

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Contributions and Acknowledgments

SR and CR contributed equally to study design and flow cytometry analyses. SR was responsible for manuscript writing and statistical analyses. PP contributed to flow cytometry experiments. TB performed slide immunofluorescence. AD provided clinical data. LML was responsible for the evaluation of bone marrow trephine biopsies. GL was responsible for the direct supervision of the study and gave final approval.

The Authors are grateful to Dr. Luca Pierelli for a critical reading of the manuscript.

Funding

This study was supported in part by A.I.R.C. (Associazione Italiana Ricerca sul Cancro).

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received November 16, 1998; accepted February 2, 1999.

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Haematologica 1999; 84:425-430 original paper

Clinical characteristics, treatment outcome and survival of 36 adult patients with primary anaplastic large cell lymphoma

GIUSEPPE LONGO, CLAUDIA FIORANI, STEFANO SACCHI, VINCENZO CALLEA,* MARCO LOMBARDO,° MASSIMO FEDERICO, CATERINA STELITANO,* FRANCESCO ANGRILLI,° DANIELE VALLISA,§ PAOLO G. GOBBI,@ FIORELLA ILARIUCCI,# ANTONIO FRASSOLDATI, MARIO PETRINI,^ VITTORIO SILINGARDI
FOR THE GRUPPO ITALIANO PER LO STUDIO DEI LINFOMI (GISL)

*Department of Internal Medicine, Oncology and Radiology Sciences, University of Modena; *Division of Hematology, Ospedale Riuniti, Reggio Calabria; °Department of Oncology and Hematology, Ospedale di Pescara; §Division of Internal Medicine, Section of Hematology, Ospedale Piacenza; @Department of Internal Medicine and Oncology, University of Pavia, IRCCS Policlinico San Matteo, Pavia; #Division of Hematology, S. Maria Nuova Ospedale Reggio Emilia; ^Haematology Unit, University of Pisa; Italy*

ABSTRACT

Background and Objective. **Although in recent years anaplastic large-cell lymphoma (ALCL) has emerged as a distinct clinico-pathological entity, a gold standard for treatment has still not been defined. Goals of our histologic, phenotypic and clinical study were to present clinical findings, treatment outcome and survival rates of a small, but highly homogeneously treated, series of patients.**

Design and Methods. **From April 1991, 36 newly diagnosed adult patients with systemic ALCL CD30+, entered a prospective non-randomized trial in one of the institutions participating in a GISL (*Gruppo Italiano per lo studio dei Linfomi*) study and were treated with a MOPP/EBV/CAD hybrid scheme. Chemotherapy (CHT) was administered every 28 days, for a total of 6 cycles. After CHT, 19 patients received radiation therapy (RT) to the site of previously involved fields. Kaplan and Meier and log-rank tests were used for statistical analysis.**

Results. **The overall complete remission rate was 78%, the partial remission rate was 6%. The overall survival rate at 74 months was 69%. No statistically significant differences in response or survival rates were noted comparing ALCL-HL and -CT subgroups, T+ Null– and B– subtypes, or ALCL-HL and -CT, with different phenotypes. In the analysis of patients with T+ Null phenotype treated with CHT+RT in comparison with B-ALCL patients who had the same treatment, we observed statistically significant differences in the survival rate (p=0.048). No prognostic factors predictive of response or survival were identified.**

Interpretation and Conclusions. **Our results show that using MOPP/ABV/CAD the results, in terms of remission rate and survival, are similar to those obtained with 3rd generation CHT regimens. The diagnosis of T and Null ALCL is the most important prognostic factor, because it is associated with a very good survival, even in patients with a high prognostic index. Finally, we believe that longer follow-ups are needed to evaluate long-term survival and toxicity with different treatments.**
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Correspondence: Stefano Sacchi, M.D., Department of Internal Medicine, Oncology and Radiology Sciences, University of Modena, via Del Pozzo 71, 41100 Modena, Italy.
Phone: international +39-059-422175 – Fax: international +39-059-424549 – E-mail: ssacchi@unimo.it

Key words: ALCL, CD30, chemotherapy, radiotherapy, survival

In 1985, Stein demonstrated the expression of the lymphoid activation antigen CD30/Ki-1 by neoplastic cells.[1] Lymphomas expressing this antigen were defined as Ki-1/CD30+ anaplastic large cell lymphoma (ALCL) and were incorporated into the updated Kiel classification as a separate entity in 1989.[2] Although several ALCL have been reported to express antigens of T-or B-cell lineage, many cases may lack lymphoid antigens (Null type) and rare cases may express both markers.[3] The *Revised European-American Lymphoma* (REAL) classification[4] limited the term of ALCL to T-and Null-cell types, including the B-cell type among the morphologic variants of diffuse large B-cell lymphoma. Four distinct histologic varieties have also been recognized, with the most frequent being the Common type (CT) and the Hodgkin's-related (HR) variety.[5-7] HR-ALCL was reported as a distinct provisional entity in the Real Classification and the term HR was replaced by Hodgkin's-like (HL). Herein we report on 36 adult patients with primary ALCL treated at a GISL (*Gruppo Italiano Studio Linfomi*) center with a MOPP/EBV/CAD hybrid regimen[8] followed by radiotherapy (RT) of the involved field when indicated.

Design and Methods

Patient population

From April 1991 to November 1997, 36 newly diagnosed adult patients with ALCL CD30+, referred to one of the Institutions participating in the GISL study were enrolled in this trial and treated with a MOPP/EBV/ CAD hybrid regimen.[8] The criteria for eligibility included a confirmed histologic diagnosis of ALCL CD30+, stage II to IV according to the Ann

Arbor system[9] or stage I with bulky disease, Karnofsky performance status (PS) over 50, age between 20 to 65 years, and normal cardiac, renal, pulmonary and hepatic functions. The characteristics of the 36 patients are listed in Table 1.

Histopathologic and phenotypic analyses

Slides from routinely paraffin-embedded tissues were stained with hematoxylin-eosin; immunophenotypic analysis was performed on paraffin-embedded sections by Apaap labeling. The diagnosis of ALCL was made according to standard diagnostic criteria,[1] including classic histologic features and reactivity of tumor cells with CD30/Ber-H2. The panel of monoclonal antibodies included CD45/LC, CD30/Ber H2, CD20/L 26, CD45/RO, CD3, CD15, EMA/E29 (DAKO, Glostrup, Denmark).

ALCL were considered as being $CD30^+$ if at least 75% of neoplastic cells stained for the CD30 antigen. Lymphomas were considered of B-cell lineage when tumor cells expressed CD20. They were considered of T-cell origin when tumor cells expressed CD3, or in the absence of $CD3^+$, expressed CD45RO, but not CD20. A lymphoma was determined to be *Null type* when no staining was obtained after testing for B and T lineage.

Staging

Staging procedures included physical examination, LDH determination, HIV, HBV and HCV antibody screening, blood cell and differential counts, liver and renal function tests, computerized tomographic scans of neck, chest, abdomen, and pelvis, and unilateral bone marrow biopsy. Patients were staged according to the Ann Arbor classification.[9]

Treatment protocol

Previously reported[8] as an aggressive regimen for patients with prognostically unfavorable, advanced Hodgkin's disease, the MOPP/EBV/CAD hybrid regimen consists of mechlorethamine (substituted in alternate cycles by CCNU), vindesine, melphalan, prednisone, followed on day 8 by epidoxorubicin, vincristine and procarbazine, and on day 15 by vinblastine and bleomycin. Chemotherapy (CHT) was administered every 28 days, for a total of 6 cycles. Drug doses and administration schedules are listed in Table 2. After CHT, 19 patients received RT to the site of previous involved fields, mainly bulky disease in the mediastinum.

Assessment of response

One month after the end of therapy re-staging was performed by physical examination, blood cell and differential counts, liver and renal function tests, LDH evaluation, CT scans of neck, chest, abdomen and pelvis and bone marrow biopsy, in case of positivity at diagnosis. Complete remission (CR) was defined as the disappearance of disease-related signs and symptoms, as well as the normalization of all previous abnormal findings. Partial remission (PR) was defined as a greater than 50% reduction of known measurable

Table 1. Characteristics of the 36 patients with ALCL.

Characteristics	*No. of patients*	*Percentage (%)*
N. of patients	36	100
Mean age, years	42	
Range	18-73	
Sex		
Male	21	58
Stage		
I and II	18	50
III and IV	18	50
Karnofsky		
50-80%	18	50
>80%	18	50
Bulky disease		
Absent	23	64
Systemic symptoms		
Absent	18	50
Extranodal sites		
Bone Marrow	3	8
Waldeyer's ring	1	3
Spleen	5	14
Liver	1	3
Lung	3	8
Stomach	3	8
Skin	2	6
Histology		
Common	20	55
HR	16	45
Phenotype		
B 10	28	
T + Null	26	72
Treatment		
Chemotherapy	17	47
Chemotherapy + radiotherapy	19	53
International Prognostic Index		
Low + low-intermediate	22	62
High-intermediate + high	14	38
LDH Level		
≤1 x Normal	20	55
>1 x Normal	16	45

Table 2. MOPP/EBV/CAD hybrid regimen: drug doses and time schedule.

Drugs	*Dose (mg/m^2)*	*Route*	*Days*	*Cycle*
Mechlorethamine	6	IV	1	cycles 1, 3, and 5, only
Lomustine	100	Oral	1	cycles 2, 4, and 6, only
Vindesine	3	IV	1	
Melphalan	6	Oral	1-3	
Prednisone	40	Oral	1-14	
Epidoxorubicin	40	IV	8	
Vincristine	1.4	IV	8	
Procarbazine	100	Oral	8-14	
Vinblastine	6	IV	15	
Bleomycin	10	IV	15	

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disease with disappearance of the systemic symptoms. No response (NR) was defined as less than PR. In 5 patients, with residual masses in the mediastinum, magnetic resonance imaging (MRI) and gallium-67-citrate single-photon emission computed tomography (67 Ga SPECT) were performed. These patients were considered to be in CR when repeated CT scans and/or MRI and/or 67 Ga SPECT did not show changes for at least 12 months.

Statistical methods

All data were analyzed with the Statistical Package for Social Sciences (SPSS).[10] The overall survival was measured from the date of diagnosis to death from any cause or date of last follow-up evaluation. Survival rates were estimated by the method of Kaplan and Meier.[11] Ninety-five percent confidence intervals can be approximated as the life-time table estimates ±1.96 SD. The log-rank test was used whenever appropriate to assess the significance of differences between groups.[12]

Results

At the time of this analysis, the median follow-up period was 35 months, and the maximum follow-up was 7.3 years.

Clinical presentation

The main clinical findings of the 36 patients with ALCL are shown in Table 1. The male/female ratio was 1.4: 1 and the mean age 42 years, but there was a very large range (14-79 y). B symptoms, advanced stages and CT histologic variant of ALCL were observed in about 50% of patients; bulky disease, mainly in the mediastinum was found in 36%. We noted some differences in clinical pictures between patients with CT and HL histologic subtypes and between patients with T + Null and B phenotype subgroups, but these were not statistically significant.

Response to treatment

The complete response rate after CHT was 64% (23/36) and the partial response rate was 22% (8/36), with a major response rate (CR + PR) of 86%; 14% (5/36) of the patients showed no response. After CHT, 19 patients were treated with radiation therapy (RT). One month after RT, 84% (16/19) were in CR and 5% (1/19) were in PR; NR was observed in 11% (2/11) of patients. Thus, the overall response rate in our series of 36 patients after CHT±RT was as follows: CR 28 patients (78%), PR 2 patients (6%) and NR 6 patients (16%) (Table 3). No significant differences were observed between patients with ALCL-HL and -CT, since 81% and 90% obtained CR+PR, respectively (Table 3). It is noteworthy that the 3 patients who relapsed had the ALCL-CT subtype. Nor were any significant differences observed between patients with T+ Null- and B- subtypes, since 88% and 80% obtained CR+PR, respectively (Table 3). Finally, we did not observe statistically significant differences between patients with ALCL-HL and -CT, with different phenotypes (Table 3).

Table 3. Treatment response and survival rate, overall, by histologic and phenotypic subtypes, and by histologic subtypes expressing different phenotypes.

	Patient No.	*CR (%)*	*PR (%)*	*NR (%)*	*Survival Rate (%)*	*95% CI*
Overall	36	28 (78)	2 (6)	6 (16)	69	52-76
HL	16	11 (68)	2 (12)	3 (20)	68	47-89
CT	20	17 (85)	1 (5)	2 (10)	70	43-97
T and Null	26	22 (85)	1(4)	3 (11)	75	56-95
B	10	6 (60)	2 (20)	3 (30)	56	25-87
HL (B-cell)	5	3(60)	1(20)	1(20)	60	19-100
HL (T+Null-cell)	11	8(73)	1(9)	2(18)	72	47-97
CT (B-cell)	5	3(60)	1(20)	1(20)	53	6-100
CT (T+Null-cell)	15	14(93)	0(0)	1(7)	77	46-100

Survival

The survival rate for all the 36 patients at 74 months was 69% (95% CI, 52-76) (Figure 1 and Table 3). We did not find any statistical differences (p=0.4) between survival rates of ALCL-HL and -CT patients, 68% (95% CI, 47-89) and 70% (95% CI, 43-97) respectively (Figure 2 and Table 3). Comparing the group of patients who received only CHT with those who had CHT + RT we did not find statistically significant differences (Figure 3). Figure 4 shows the survival curves of Null+ T- and B- subtype ALCL patients. The differences between subgroups in overall survival were not significant (p=0.12), since B and T+ Null patients showed, at 60 and 81 months, an overall survival rate of 56% (95% CI 25-87) and 75% (95% CI 56-95), respectively. Although no statistically significant differences were observed, there was a ten-

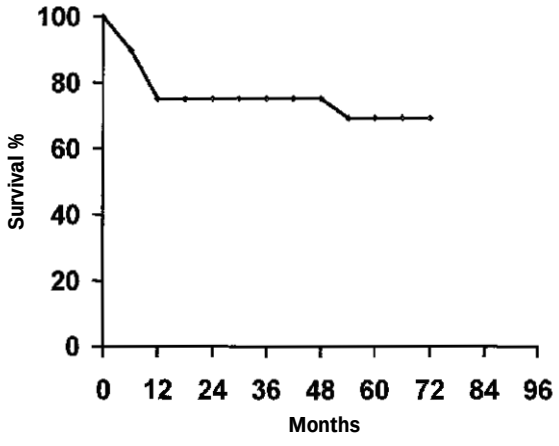

Figure 1. Overall survival curves of 36 ALCL patients.

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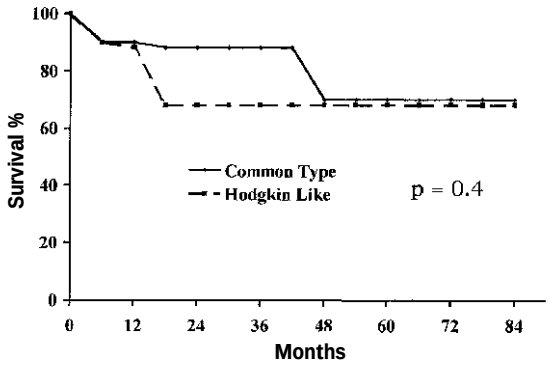

Figure 2. Comparison of survival curves of patients with ALCL-HL and -CT (p=0.4).

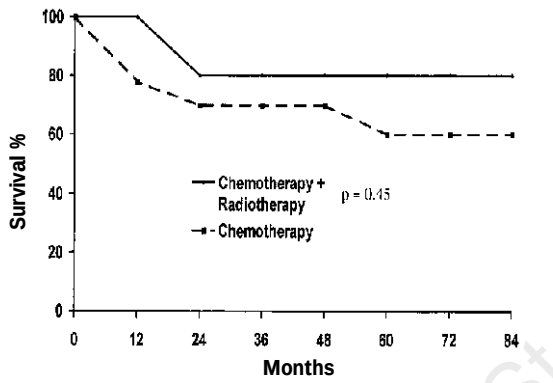

Figure 3. Comparison of survival curves of patients treated with CHT alone and those treated with CHT + RT (p=0.45).

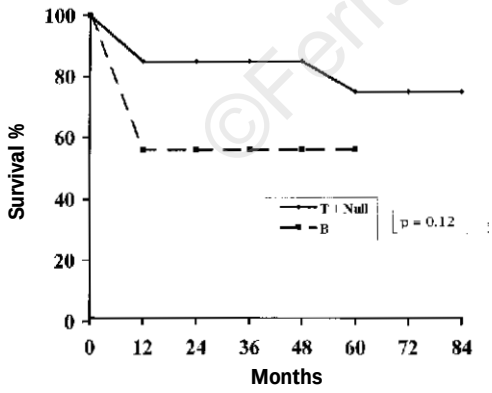

Figure 4. Comparison of survival curves of patients with T + Null- and B-phenotypic subgroups (p=0.12).

dency towards poor survival rate in B-ALCL patients. Analyzing survival distribution for phenotype adjusted for RT (Figure 5), we observed that T+Null patients treated with CHT+RT had an overall survival

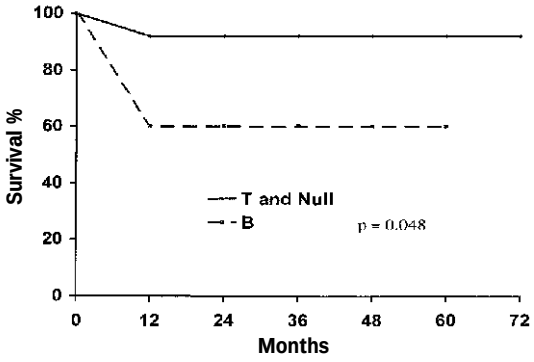

Figure 5. Comparison of survival curves of patients with T + Null- and B-phenotypic subgroups treated with CHT+RT (p<0.048).

rate of 92% (95%CI 79-100) at 74 months, while the B subgroup, treated with the same scheme, had a survival rate of 60% (95% CI 40-100) at 62 months (p=0.048). Finally, we did not find statistically significant differences between patients with HL- and CT-ALCL, with different phenotypes.

Factors predictive of response and survival

The characteristics of patients and their disease are usually considered as prognostic factors: age, presence of B symptoms, I-II versus III-IV stages according to Ann Arbor system,[9] performance status, LDH level, bone marrow involvement, and bulky disease were correlated to response to treatment and survival in univariate analysis. We did not find any correlation between these parameters and treatment outcome or overall survival rate. The *International Prognostic Index* (IPI)[13] developed for aggressive lymphomas in general did not predict survival in our group of patients.

Discussion

In recent years ALCL has emerged as a distinct clinico-pathologic entity, although it is a relatively uncommon disease. In the GISL registry, ALCL accounts for 2.8% of 2871 cases (85 patients) and 4.5% of 1398 cases (63 patients) of NHL enrolled in clinical trials since 1988. Our knowledge of ALCL is limited because of the small number of patients analyzed in the single series and the variety of treatments utilized.[14-29] On the basis of treatment strategies adopted by GISL in January 1991, our 36 adult patients with primary ALCL were treated with a MOPP/EBV/CAD hybrid regimen. This aggressive regimen, originally designed for patients with prognostically unfavorable, advanced HD, was subsequently adopted by GISL for patients with ALCL CD30+, because of encouraging results achieved in HD. The main clinical characteristics of our patients at the time of diagnosis differ slightly from those given in other reports. The male/female ratio was 1.4:1 and the mean age 42 years, but there was a

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very large range (14-79 y). B symptoms, advanced stages and CT histologic variant of ALCL were observed in about 50% of patients; bulky disease, mainly in the mediastinum was found in 36%. The overall major response rate in our patients was 84%, which is similar to the rate obtained in other recent series.[27,29] No statistically significant differences in response and survival rate were noted comparing CT- and HL-subgroups, T+ Null- and B- subtypes, and ALCL-HL and -CT, with different phenotypes. Regarding the overall survival, we obtained a rate of 69% at 74 months, again similar to that observed in other ALCL series.[27,29] In particular we did not observe statistically significant differences in survival between patients treated with CHT + RT or with CHT alone. There is, however, a trend towards better survival in patients treated with CHT + RT than in those treated with CHT alone and in patients with T + Null-ALCL in comparison with those with B-ALCL. In the analysis, combining patients with T + Null phenotype treated with CHT + RT in comparison with B-ALCL patients who had the same treatment, we observed statistically significant differences in the survival rate. Our data, showing a tendency towards better results in patients with T+ Null phenotype, support the recent proposition of the REAL classification to consider B-ALCL as a variant of diffuse large B-cell lymphoma, and also support the hypothesis that RT could improve the prognosis of these NHL patients. Further, breaking down patients by histologic subtypes, this regimen, originally designed for aggressive HD appears as effective in ALCL-CT, universally recognized as NHL, as in ALCL-HL, a borderline entity lying midway between NHL and HD.[30] By univariate analysis no factors predicted treatment outcome or survival although other authors[27,29] have found some parameters to be prognostic factors for survival. In agreement with the *Non-Hodgkin's Lymphoma Classification Project*[31] we found that the IPI, developed for aggressive lymphomas in general, did not predict treatment outcome or survival. The diagnosis of T and Null ALCL was the most important prognostic factor, because it was associated with a very good survival, even in patients with a high prognostic index. The good outcome of T + Null-ALCL patients emphasizes the importance of phenotypic studies and does not support the use of intensive chemotherapy with autologous stem cell support.

The MOPP/EBV/CAD hybrid regimen is a valid and relatively well tolerated scheme for the treatment of ALCL, producing results equivalent or superior to 3rd generation regimens of CHT. We did not observe secondary myelodysplasia (MDS), leukemia or second cancers in any patient after a median follow up of 35 months (maximum 7.3 years). Gobbi *et al.*,[32] using the same scheme for the treatment of 145 patients with advanced HD, observed second MDS, secondary lung cancer and colorectal cancer in 2.1%, 0.7%, and 1.4% of patients, respectively, with a median follow-up of 66 months. Recently, Zinzani *et al.*[30] reported the results of a randomized trial of ABVD versus MACOP-B with or without RT in 40 ALCL-HL patients. They showed that, in terms of CR and relapse free survival, patients respond in an equivalent way to both treatments. In the 21 patients treated with ABVD the CR rate was 91% and the median survival rate at 37 months was 90%. ABVD seemed to induce higher response rate in ALCL-HL than in advanced HD, in which a CR rate of 82% was observed.[33] The 11 ALCL-HL patients with a T+ Null phenotype enrolled in our trial had a CR rate of 73% (major response 82%) and an overall survival rate of 72% at 70 months. We agree with this idea of testing less aggressive regimens, such as ABVD, in order to avoid the risk of over-treating this group of patients with a surprisingly good survival. We do, however, believe that longer follow-ups are needed to evaluate long-term survival and toxicity with different treatments, such as aggressive or standard protocols for HD or 3rd generation CHT regimens for NHL.[34,35]

Contributions and Acknowledgments

All the authors contributed to the design of the study and to recruiting patients. GL, CF and SS performed the analysis and interpretation of data, and wrote the paper. All the authors gave their critical contribution to the manuscript and approved its final version. VS is a GISL supervisor.

Our thanks to Dan L. Longo, MD; National Institute on Aging/NIH Gerontology Research Center, Baltimore, USA, for his critical reading of the manuscript.

Funding

This work was supported by a MURST (60%) grant and the Fondazione Ferrata-Storti.

Disclosures

Conflict of interest: none

Redundant publications: preliminary results of this study were presented at the XXXVI Congresso Nazionale Società Italiana di Ematologia, Catania, October 1997.

The initial follow-up of 10 out of the 36 patients was published in 1995 (ref. #24).

Manuscript processing

Manuscript received November 17, 1998; accepted January 27, 1999.

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Haematologica 1999; 84:431-436 decision making and problem solving

Splenectomy in hematology. Current practice and new perspectives

UMBERTO BACCARANI,* GIOVANNI TERROSU,* ANNIBALE DONINI,* FRANCESCO ZAJA,°
FABRIZIO BRESADOLA,* MICHELE BACCARANI°
**Chair and Division of Surgery, Department of Surgery, and °Chair and Division of Hematology, Department of Clinical and Morphologic Research, Udine University Hospital and School of Medicine, Udine, Italy*

ABSTRACT

Background and Objective. **Progress and changes in the management of blood diseases, in surgery and in videotechnology stimulate a critical reappraisal of splenectomy in hematology.**

Design and Methods. **We have collected information on the current practice of splenectomy in hematology in Italy and we have reviewed the results of a new technique of laparoscopic splenectomy (LS).**

Results**. Current splenectomy practice: the current practice in Italy is to offer splenectomy as front-line treatment for hereditary spherocytosis and as second-line for idiopathic thrombocytopenic purpura (ITP) and hemolytic anemia. Splenectomy is also offered in selected cases of leukemia and lymphoma but is going out of practice for hairy cell leukemia and Hodgkin's disease. The number of splenectomies that are performed every year is estimated to be higher than 10 per 10^6 persons (more than 500 cases per year). Laparoscopic splenectomy (LS): more than 700 cases of LS have been reported so far, for thrombocytopenia (470 cases) as well as for many other hematologic indications. The procedure carries a mortality of 0.8%, and a complication rate of 12%. Time spent in the operating theater ranges from 1.5 to 4 hours, blood transfusion requirement is minimal and the mean post-operative hospital stay is 3 days.**

Interpretation and Conclusions. **Although a prospective comparison is not available, the results of LS compare favorably with the results of classic open splenectomy, so that LS is likely to become the technique of choice especially when the spleen is small, as in ITP. LS can also have some advantages in other cases of splenectomy, including splenomegaly for leukemia and lymphoma. These data and suggestions should stimulate and renew a discussion about splenectomy in hematology, with the purpose of establishing evidence-based guidelines.**
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Key words: splenectomy, laparoscopy, leukemia, lymphoma, anemia, thrombocytopenia

Correspondence: Umberto Baccarani, M.D., Department of Surgery, Udine University Hospital, 33100 Udine, Italy. Phone: international +39-0432-559557 – Fax: international +39-0432-559555.

For many years splenectomy was the main point of contact between hematology and surgery. The indications for splenectomy in hematology are numerous, from diagnostic and staging purposes to splenic rupture due to infarction, from anemia or thrombocytopenia to leukemia or lymphoma. These indications were generated over different historical periods. Many of them are soundly based on common sense and practice, but rarely on evidence. Over time the indications for splenectomy in hematology have undergone some changes,[1] depending on modifications and progress in the management of malignant and non-malignant blood diseases. Major examples are Hodgkin's disease (HD) and hairy cell leukemia (HCL) where new treatment strategies and new therapeutic agents have minimized recourse to splenectomy.[2-5] The use of splenectomy is also influenced by the frequency and the severity of its complications. Late complications mainly involve the immune system and host defence, being infectious, and advise against splenectomy in children.[6-10] Short term complications are related to the underlying disease and the patient's clinical and hematologic conditions, but also to surgical technique and skill. Progress in surgery has made it possible to perform splenectomy under videolaparoscopic control avoiding laparotomy.[11-14] It may, therefore, be opportune to reexamine this meeting point between hematology and surgery and work out evidence-based analysis with the purpose of reaching a consensus and proposing guidelines. This review is propedeutic to the analysis and stimulatory to the discussion, reporting on the splenectomy policies that are currently practiced in Italy and illustrating the technique and the results of videolaparoscopic splenectomy. Data and discussion are limited to splenectomy in adults.

Splenectomy practice in Italy: an overview

To collect information on the current practice of splenectomy in hematology in adults, we addressed a simple questionnaire to 52 centers of which 41 were Divisions of Hematology and 11 were Divisions of Internal Medicine or Oncology with a known interest in hematology. Centers were located at University or General Hospitals all over Italy. Fourty-seven answers (90%) were received. The questionnaire asked the

respondents to identify the current main indications for and contraindications to splenectomy in chronic idiopathic thrombocytopenic purpura (ITP), hemolytic anemia, primary myelofibrosis (MF), non-Hodgkin's lymphomas (NHL), chronic lymphocytic leukemia (CLL), HCL and HD. In all the answers, a distinction was made between hereditary spherocytosis (HS) and chronic autoimmune hemolytic anemia (AHA). Moreover, the answers clarified whether, in any given condition, splenectomy was recommended always, sometimes, never or exceptionally.

All 47 Institutions declared using splenectomy in HS, AHA and chronic ITP (Table 1). In HS all centers but two advise splenectomy as front-line treatment without any major contraindications. In chronic AHA and in chronic ITP splenectomy is mainly advised in case of resistance to treatment or when the course is heavily dependent on chronic treatment. Major contraindications, as identified by responding centers, are advanced age (42% of responding centers for AHA, 60% for ITP), cold IgM autoantibodies (9%), HBV or HCV positivity (7%), immunodeficiency or HIV positivity (4%) and systemic autoimmunity (9%).

In primary MF with liver/spleen myeloid metaplasia (Table 1), splenectomy is no longer recommended in 13/47 centers (28%). Major indications are splenomegaly (68%), anemia (21%) and symptoms (47%). Major contraindications are advanced age (42% of responding centers), a high platelet count (39%) and disease acceleration or progression (27%). Splenectomy for NHL, CLL and HCL (Table 1) is currently not practised by 2%, 28% and 51% of the centers, respectively. Major indications are splenomegaly (41%, 42% and 21%), primary splenic lymphoma or isolated splenomegaly (67% in NHL), and residual or refractory splenomegaly (20%, 28% and 30%).

In HD, splenectomy is out-of-practice in 32/47 centers (68%) (Table 1). In the remaining 15 centers splenectomy is practised only for the surgical staging of a few, selected early stage cases and sometimes also for the management of an isolated splenomegaly.

To summarize, splenectomy is currently indicated as front-line treatment in HS, irrespective of the degree of anemia, because only two of the 47 centers answered that the recommendation for splenectomy depended on Hb level. It is recommended as second-line treatment in chronic AHA and chronic ITP, when medical treatment fails or treatment duration and toxicity are not acceptable. Interestingly, disease severity and duration were not indicated, while five centers pointed out that splenectomy can be required to eliminate or reduce the anxiety of living with ITP or AHA. In MF, in lymphomas and in chronic lymphoproliferative disorders splenectomy is no longer used as front-line treatment. However, it is still recommended in MF when splenomegaly is massive and symptomatic, and in NHL and CLL, mainly when spleen volume is large or the spleen is refractory to treatment, with anemia or thrombocytopenia. The case is different with HCL, where 50% of the institutions have abandoned splenectomy completely, due to the increasing availability of very effective medical treatments[4] and with HD, where staging laparotomy and splenectomy have fallen out of use, either because of the improvement in imaging techniques or because of the increased effectiveness of other treatments.[2,3,15] Fear that splenectomy can increase the rate of late second tumors[16-18] may also have contributed to the abandoning of splenectomy.

Laparoscopic splenectomy

Splenectomy was traditionally performed through a midline or left subcostal laparotomy (open splenectomy, or OS). The development of new video-technology combined with advances in surgical instrumentation has allowed the surgeon to enter the peritoneum through multiple tiny incisions of the skin under direct visualization offered by a fiber-optic scope and a videocamera connected to a TV screen. This new laparoscopic technique allows several surgical procedures to be performed, for example cholecystectomy, appendectomy, hernia repair, gastric fundoplication, adrenalectomy.[19] In 1992, Carroll *et al.* in Los Angeles were the first to use laparoscopy for splenectomy.[11] Since then, several centers worldwide have adopted this new approach. The abdomen is insufflated with CO_2 to create a virtual chamber in the peritoneal sac. Special hollow devices, called trocars, are used to introduce the fiber-optic scope and the laparoscopic instruments into the peritoneal cavity. The operation can now be performed entirely laparoscopically. Usually 4 to 5 trocars, located in the upper abdomen, are enough to perform a laparoscopic splenectomy (LS). The operation starts with dissec-

Table 1. A summary of the major indications for splenectomy, based on current practice in 47 Italian hematologic centers.

		Chronic						
	HS %	*AHA* %	*ITP* %	*MF* %	*NHL* %	*CLL* %	*HCL* %	*HD* %
Always	95	0	0	0	0	0	0	0
Sometimes	5	100	100	72	98	72	49	32
Never or exceptionally	0	0	0	28	2	28	51	68
Resistance to treatment	–	95	79	2	0	8	30	0
Treatment dependence	–	6	23	0	0	0	0	0
Bleeding	0	0	17	0	0	0	0	0
Severe thrombocytopenia	0	2	30	8	15	15	4	0
Severe anemia	5	0	0	21	15	19	2	0
Spleen volume	0	0	0	68	41	42	21	0
Residual or refractory splenomegaly	–	–	–	0	20	28	30	0
Isolated splenomegaly	–	–	–	–	67	2	0	4
Symptoms	0	0	0	47	6	4	2	0
Pathologic staging	–	–	–	–	2	–	–	30

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tion of the spleno-colic ligament at the lower pole of the spleen. Mobilization of the spleen proceeds upward by dissection of the spleno-renal ligament and of the spleno-phrenic attachments at the upper pole of the spleen. The splenic vein and artery are now best managed with the aid of a mechanical linear stapler loaded with vascular cartridges. When the spleen is completely free in the abdominal cavity, it can be retrieved either by morcellation into a strong plastic bag or full-size through enlargement of a trocar incision or through an accessory suprapubic incision, when pathologic examination of the whole organ is required. The use of a fiber-optic scope connected to a TV screen allows the surgeon to perform the entire procedure under direct vision. Moreover video-technology instrumentation permits magnification of the anatomic structures. Liver, abdominal and retroperitoneal lymph nodes, accessory spleens and pelvic structures can also be well visualized during the laparoscopic procedure. A careful abdominal exploration with multiple biopsies can be performed allowing complete disease staging when necessary.

LS is a technically demanding procedure and surgeons follow a well-defined learning curve. The experience of the surgical team is the main determinant of the duration of surgery. The reported operating theater time ranges between 1.5 and 4 hours.[13,20-32] Other important factors are spleen volume, the presence of an accessory spleen and obesity. LS for HD clearly requires a longer time because of the staging procedure. The accuracy of LS for staging is difficult to evaluate. However, based on our experience of 15 cases of LS and 40 cases of OS[32] there is no suggestion that LS is less accurate than OS and we think that the magnified view provided by the laparoscope can even improve the detection of small foci of the disease. For the same reason, LS is comparable to OS for the detection of small accessory spleens. This is particularly important in chronic ITP, where failure to recognize and remove an accessory spleen can be responsible for the persistence of thrombocytopenia.[10] With LS accessory spleens are found in 5 to 25% of cases, and the same frequency is reported with OS.[12,23-24,26-28,31,33-34]

Intraoperative blood loss ranges from less than 50 mL up to 500 mL, but in some cases a blood loss of more than 1000 mL has been reported, requiring conversion to laparotomy. However, conversion may also be recommended depending on the rapidity of the blood loss and on the patient's age and general conditions.

The post-operative course after LS averages less than 4 days, with 50% of patients being advanced to oral diet the day after surgery.[13,20-40] The duration of the post-operative stay is obviously related to the frequency and severity of post-operative complications. These are shown in Table 2, based on 500 reported cases with available information.[13, 20-40] Major (6%) and minor (6%) complications were reported in 60 of 500 cases or 12% overall. There were four deaths; two due to hemorrhage associated with persistent severe thrombocytopenia,[26] one to cytotoxic treatment,[26] and one to myocardial infarction.[22] It is worth noting the low frequency of pleuropulmonary and pleuric complications (2.4% and 1%, with one case of diaphragmatic perforation).

Table 2. Mortality and morbidity of laparoscopic splenectomy in 500 reported cases. Four patients died because of: CNS bleeding due to persistent severe thrombocytopenia (2 cases), complications related to cytotoxic treatment for NHL (1 case), myocardial infarction (1 case). Complications were seen in 60 cases (12%), and were reported to be major in 30 cases (6%).

Deaths	4	0.8%
Major complications		
- pleuropulmonary	12	2.4%
- deep venous thrombosis	5	1.0%
- bleeding	5	1.0%
- subphrenic hematoma or abscess	4	0.8%
- herniation at trocar site	2	0.4%
- diaphragmatic perforation	1	0.2%
- transitory ischemic attack	1	0.2%
Total	30	6.0%
Minor complications*	30	6.0%

**Including wound infection (0.8%), transient serum amylase elevation (0.6%), back pain, hematuria from Foley catheter, subcutaneous emphysema, prolonged ileus, atrial fibrillation, transient pericardial effusion, pain at trocar site, urinary retention, scrotal edema and paresthesia.*

Not all the complications and the problems that can occur during LS can be managed laparoscopically. In some cases, laparotomy is required and LS is converted to OS. The reported conversion rate for hematologic diseases ranges from none to 18%,[13,20-40] depending on the experience of the surgical team and on the degree of splenomegaly, which is associated with more extensive adhesions and particularly with hemorrhage from the splenic vein or artery at the hilum or with bleeding from the parenchyma or short gastric vessels. Extensive bleeding is by far the commonest cause of conversion. A loss of more than 1000 mL of blood demands immediate conversion in any case.

The operative costs, including instrumentation, operating theater, hospitalization and treatment of complications, have been estimated to range between 7,000 and 18,000 US dollars for LS vs 9,000 to 14,000 US dollars for OS.[25-28, 31]

There are no studies prospectively comparing the results of OS and LS and it is difficult that such studies will be ever done. However, in several reports,[22-28, 31-32] an attempt was made to provide the data of LS together with data from prior OS, so as to make a comparison possible. These data are summarized in Table 3, and they help to form a clear and consistent picture of LS. The operating theater time used for LS is distinctly longer than that for OS (median 196 vs 121 minutes), the estimated blood loss is similar, the frequency of the detection of an accessory spleen is

Table 3. A summary of nine reports comparing laparoscopic splenectomy (LS) with open splenectomy (OS). All the cases reported by Schlinkert *et al*.[23] and Watson *et al*.[31] and about 50% of all the other cases concerned treatment of ITP, with the exception of the report by Baccarani *et al.*,[32] that concerned HD. The differences (asterisk, p-value < 0.05) were calculated in the original reports. NR = not reported.

	Number of cases		Incidence of accessory spleen (%)		Mean operating theater time (min)		Mean estimated blood loss (mL)		Mean time to liquids (days)		Mean post-operative stay (days)		Complications %		Mortality %	
References	*LS*	*OS*	*LS*	*OS*	*LS*	*OS*	*LS*	*OS*	*LS*	*OS*	*LS*	*OS*	*LS*	*OS*	*LS*	*OS*
Rhodes 1995	24	11	NR	NR	120*	75*	NR	NR	NR	NR	3.0*	7.0*	8	27	4	0
Schlinkert 1995	7	14	0	14	154*	68*	NR	NR	0.7*	2.6*	2.1*	5.0*	0	21	0	0
Brunt 1996	26	20	11	5	202*	134*	222	376	1.4*	4.1*	2.5*	5.8*	23	30	0	0
Smith 1996	10	10	NR	NR	261*	131*	NR	NR	1.9*	4.4*	3.0*	5.8*	0	20	0	0
Watson 1997	13	47	15	6	88	87	NR	NR	NR	NR	2.1*	13.4*	0	19	0	0
Diaz 1997	15	15	20	20	196*	116*	385	359	NR	NR	2.3*	8.8*	7	13	0	0
Friedman 1997	63	74	17	13	153	121	259	437	1.5*	3.2*	3.5*	6.7*	4	17	0	3
Glasgow 1998	52	28	13	25	196*	156*	274	320	2.0*	4.3*	4.8*	6.7*	10	14	6	0
Baccarani 1998	15	40	NR	NR	202*	144*	NR	NR	1.9*	3.2*	4.4*	6.7*	6	28	0	0
Total	225	259													1.5	0.7
Median			13	13	196	121	266	367	1.7	3.6	3.0	6.7	6	20	–	–

**p-value < 0.05.*

similar, the *time-to-liquids* and the post-operative stay are significantly shorter, and complications are likely to be less frequent. Mortality is low with both operations.

The indications for LS are not yet defined. Several hundred cases have been reported so far (Table 4) with the great majority having been carried out in chronic ITP, reflecting the frequency of the disease, the large consensus on splenectomy[10] and the small volume of the spleen, hence the convenience of LS, where bleeding can be controlled as carefully as with OS and the post-operative course is short and uncomplicated. LS has also been performed for several other hematologic indications (Table 4), including leukemia and lymphoma. A recent study by Decker *et al*.[40] emphasized the feasibility and safety of LS for blood malignancies reporting that post-operative morbidity and mortality were not greater than in patients who were operated on for anemia or thrombocytopenia, although the time spent in the operating theater was longer, conversion to OS was more frequent, and more blood transfusions were required. Probably, the major problem is the volume of the spleen; a huge splenomegaly implies older disease with more clinical problems, more adhesions, more bleeding and more technical difficulties. Table 5 shows the data of 48 cases of LS that were performed in Udine, divided according to the degree of splenomegaly.[41] There were 14 cases of splenomegaly, including NHL (8 cases), CLL (1 case), MF (2 cases) and HS (3 cases), with a median spleen weight of more than 2000 g. The main difference with the 34 cases of LS with no splenomegaly (i.e. with a spleen weight of less than 500 g) concerned the operating theater time (168 vs 115 min), blood loss (504 vs 226 mL), blood transfusions, that were required in 5 of 14 cases of splenomegaly but in none of the 34 cases without splenomegaly, and the time to oral diet (1 vs. 2 days). The rate of conversion to OS (7% and 3%), post-operative complications (7% and 6%) and post-operative hospital stay (5 and 4 days) were similar.

Table 4. Hematologic indications for laparoscopic splenectomy: literature data.

	No. of cases
Chronic idiopathic thrombocytopenic purpura	437
HIV related thrombocytopenia	33
Hereditary spherocytosis	63
Hodgkin's disease (staging)	59
Chronic autoimmune hemolytic anemia	56
Non-Hodgkin's lymphoma	29
Chronic lymphocytic leukemia	16
Myeloproliferative disorders, including myelofibrosis	11
Hairy cell leukemia	8
Sickle cell disease	4
β thalassemia	2
TOTAL	718

Conclusions

LS is likely to be the intervention of choice when splenectomy is indicated in a patient with a small spleen and with uncomplicated disease. The prototype disease is chronic ITP, in which it has been shown that thrombocytopenia does not cause any particular or unexpected difficulty. For other indications and in any case of massive splenomegaly the difficulties that are encountered with LS are the same as those with OS. The mortality and the frequency of the complica-

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Table 5. Comparison of laparoscopic splenectomy for *normal sized* spleen (spleen weight less than 500 g) and for splenomegaly (spleen weight more than 500 g). All the patients were operated on at the Department of Surgery, Udine University.[41] Values are mean ± SD.

	Splenomegaly (> 500 g)	*Normal sized spleen (< 500 g)*
No. of cases	14	34
Spleen weight (g)	2350±1311	167±86
Conversion to open splenectomy	1 (7%)	1 (3%)
Operation theater time (minutes)	168±67	115±53
Intra-operative estimated blood loss (mL)	504±352	226±220
Blood transfusions		
no. of cases requiring blood transfusion	5 (36%)	0
mean number of units per patient	2.6	–
Clear to oral diet (days)	2.0±1.0	1.0±0.5
Post-operative complications	1 (7%)	2 (6%)
Post-operative hospital stay (days)	5±2	4±2

tions are likely to be identical, depending on disease, patient, specific surgical experience, and medical care before and after the operation. Currently, we practice LS in all cases, bearing in mind that a conversion to OS can be decided and performed whenever required in the operating theater. In other institutions the practice is different. The questionnaire that was sent to the hematologic centres included a question concerning the operative technique. Only one center answered that all the operations were LS, while in 28 centers (59%) all the splenectomies are still performed according to the classic open technique. In the remaining 17 centers (37%) both techniques, OS and LS, are in use and in these centers the major contraindications to the laparoscopic technique were reported to be spleen volume (82% of centers), low platelet count (41%), staging (41%) and advanced age (18%). MF (53%), CLL (35%) and NHL (35%) were also indicated as contraindications to LS. We do not share this opinion, but clearly more experience must be gained and more evidence provided, before LS gains a larger consensus. Finally, it is also interesting and important to have an estimate of the size of the demand. Our questionnaire asked the centers to provide an estimate of the numbers of splenectomies that are expected to be done yearly. The total ranges from 175 to 210 for chronic ITP, from 86 to 113 for NHL, from 54 to 89 for HS and chronic AHA, from 40 to 50 for CLL, from 30 to 60 for MF, from 21 to 28 for HD and from 13 to 19 for HCL. Overall, about 500 splenectomies (range 419 to 568) are performed yearly in the responding centers. This is obviously an underestimate of all the splenectomies that are performed in Italy, because the questionnaires were sent only to hematologic centers, and five did not respond. Therefore, over 500 splenectomies per year, or approximately 10 splenectomies per 10^6 persons per year are likely to be done in Italy for hematologic indications. We think that a consensus on indications and techniques is worth developing, also taking into account the progress in the management of HD[2, 3, 15-18] and HCL,[4] the uncertainties in the treatment of chronic ITP,[10,42] and the recent analysis of splenectomy and risk of blastic transformation in MF.[43] It may be difficult to reach a consensus and to propose guidelines based only on retrospective reviews and on the confrontation of individual opinions and institutional practices. The establishment of a prospective splenectomy registry covering a 2 or 3-year period would probably be more appropriate.

Contributions and Acknowledgments

UB collected and analyzed the data and wrote the report, which was critically reviewed by all the other authors. GT and AD were responsible for the surgical program and data. FZ was responsible for medical program and data. The authors are listed in an order reflecting their individual contribution to the study. FB and MB were responsible for the study, reviewed the manuscript, and are listed as last co-authors.

The kind co-operation of all Colleagues who answered the questionnaire is gratefully acknowledged.

Disclosures

Conflict of interest: none.

Redundant publications: the surgical data from Udine were reported in a paper published in the Archives of Surgery (1998) and in one in press in Surgery. Both papers are cited in the reference list.

Manuscript processing

Manuscript received November 10, 1998; accepted January 25, 1999.

Reprints: Editorial Office

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Haematologica 1999; 84:437-445 decision making and problem solving

Cancer and venous thromboembolism: an overview

PAOLO PRANDONI, ANDREA PICCIOLI, ANTONIO GIROLAMI
Department of Medical and Surgical Sciences, Second Chair of Internal Medicine, University of Padua Medical School, Padua, Italy

ABSTRACT

Background and Objective. **Although the relationship between malignant diseases and venous thromboembolism has been convincingly demonstrated, the clinical implications of this association still have to be thoroughly elucidated. The aim of this study was to review briefly the mechanisms by which cancer may induce the development of thrombosis and to analyze critically the most recent clinical advances in this field.**

Evidence and Information Sources. **The material examined in the present review includes articles published in journals covered by the Science Citation Index® and Medline®.**

State of the Art. **Neoplastic cells can activate the clotting system directly, thereby generating thrombin, or indirectly, by stimulating mononuclear cells to synthesize and express various procoagulants. Cancer cells and chemotherapeutic agents can injure endothelial cells, thereby intensifying hypercoagulability. Currently, primary prevention of venous thrombosis should be considered for cancer patients during and immediately after chemotherapy, when long-term indwelling central venous catheters are placed, during prolonged immobilization from any cause, and following surgical interventions. Secondary prevention of recurrent venous thromboses usually necessitates long-term anticoagulation. In some patients with cancer the condition is resistant to warfarin, and long-term adjusted high-dose heparin is required. The diagnosis of venous thromboembolism may help to uncover previously occult carcinoma by prompting a complete physical examination and a few routine tests.**

Perspectives. **Further investigations are required to evaluate the cost-benefit ratio of extensive diagnostic screening for occult malignancy in all patients presenting with idiopathic venous thromboembolism, and to explore the potential of low molecular weight heparins for improving survival in patients with cancer.**
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Key words: thrombosis, venous thromboembolism, cancer, anticoagulation, heparin, low molecular weight heparin, warfarin, chemotherapy

Correspondence: Paolo Prandoni, M.D., Department of Medical and Surgical Sciences, 2nd Chair of Internal Medicine, via Ospedale Civile 105, 35128 Padua, Italy. Phone: international +39-049-8212656 – Fax: international +39-049-8212661 – E-mail: paoprand©tin.it

Since the initial observation by Armand Trousseau in 1865, numerous studies have addressed the relationship between cancer and thrombosis. Post-mortem studies have demonstrated a markedly increased incidence of thromboembolic disease in patients who died of cancer, particularly those with mucinous carcinomas of the pancreas, lung, and gastrointestinal tract.[1,2] Cohort studies of surgical patients showed that the incidence of deep venous thrombosis (DVT) was markedly higher in patients with malignant disorders than in patients with other, non-malignant diseases.[3-6] An increased risk of venous thromboembolism (VTE) is suggested by the high incidence of pulmonary embolism[7] and subclinical activation of the coagulation system in non-surgical patients with cancer.[8-11] The relationship between cancer and thrombosis is further supported by the greater risk of patients with idiopathic VTE developing overt malignancy than patients whose thrombotic episode is associated with a well recognized risk factor.[12]

This article reviews the relation between cancer and VTE and highlights some relevant clinical implications.

Pathogenesis

Pathogenetic mechanisms accounting for the development of thrombotic disorders in patients affected by cancer were described by Virchow more than a century ago. They include hypercoagulability, due to tumor cell activation of clotting, vessel wall injury, and stasis.

Hypercoagulability

Neoplastic cells can activate the clotting system directly, thereby generating thrombin, or indirectly by stimulating mononuclear cells to produce and express procoagulants.

Several different procoagulant activities have been identified from tumor cell lines, extracts or sonicates of human and animal tumors. The best characterized tumor cell procoagulants are tissue factor, an integral membrane glycoprotein which can activate the extrinsic pathway through interaction with factor VIIa, and factor X activators.[13,14] Tissue factor procoagulant activity has been identified in some acute leukemias[15] and in solid tumors of the ovary, stomach, and kidney.[16] Direct factor X activation with the

procoagulant cysteine proteinase has been found in some patients with lung, prostate, colon, breast, and kidney cancer and with leukemia.[17,18] Mucin-secreting adenocarcinomas are frequently associated with thrombosis because the sialic acid moiety can cause non-enzymatic activation of factor X to its active form, factor Xa.[19] Consequently, adenocarcinomas of the lung, pancreas, gastrointestinal tract, and ovary are often associated with venous thrombosis.[20]

Tumor cells can activate systemic coagulation by stimulating mononuclear cells to synthesize and express various procoagulant substances, including tissue factor and factor X activators. Normal monocytes and macrophages can be activated by tumor cells in the presence of lymphocytes.[21] In patients with cancer, endothelial cells may be activated by cytokines such as tumor necrosis factor and interleukin-1 or interleukin-like substances that may induce tissue factor production.[22] A peptide produced by a human bladder cancer cell line stimulates tissue factor expression in endothelial cells.[23]

Clinical manifestations of increased thrombin generation may be accentuated by down-regulation of endothelial cell counterregolatory mechanisms, such as decreased hepatic synthesis of antithrombin and protein C.[8,10,24,25] In addition, normal endothelial cell function may be disrupted by various defects in platelet function.[8,10,24,25]

The enhanced clotting activation in patients with cancer is confirmed by the demonstration of increased levels of systemic hypercoagulability markers, such as fibrinopeptide A, prothrombin fragment F1+2 and thrombin-antithrombin complexes in most patients.[25-27]

As expected, the risk of (recurrent) venous thromboembolism is higher in those cancer patients who are also carriers of thrombophilia, such as the factor V Leiden mutation.[28]

Vessel wall damage

There is increasing awareness that cancer cells can injure endothelium by direct vascular invasion, resulting in the onset of a prothrombotic state. Moreover, tumor cells may secrete vascular permeability factors which account for the extravascular accumulation of fibrinogen and other clotting proteins around tumor growth.[24-26] The adhesion of tumor cells to endothelium was evaluated *in vivo* by Naschitz and associates, who observed a complex interaction between endothelium, platelets, and tumor cells.[29] Direct vessel wall injury, in association with rheologic abnormalities and catheter-associated thrombin formation, is most likely the explanation for the occurrence of the upper extremity DVT arising as a complication of central venous lines.[30] Among mechanisms responsible for thrombotic events arising during the use of chemotherapeutic drugs, vascular endothelium damage probably plays a major role besides the reduction in the plasma concentration of natural anticoagulants.[31-34]

Venous stasis

Venous stasis predisposes to venous thrombosis by preventing activated coagulation factors from being diluted and cleared by normal blood flow. Moreover, hypoxic damage to endothelial cells due to stasis may produce prothrombotic alterations. Venous stasis develops as a consequence of immobility in severely debilitated cancer patients, in conjunction with cancer surgery, or as a result of venous obstruction due to extrinsic vascular compression in patients with bulky tumor masses.[35]

Clinical implications

Search for occult malignancies in patients with idiopathic VTE

A number of studies have examined the relationship between DVT and the subsequent development of cancer.

In four studies, the incidence of newly diagnosed malignancy in patients with suspected VTE was compared with that in patients whom this diagnosis was excluded by normal objective diagnostic tests.[36-39] In all four studies the risk for new malignancy was higher among the patients with confirmed venous thromboembolism (Table 1).

Other studies compared the development of cancer in patients with apparently idiopathic VTE (no known associated risk factors) versus secondary VTE (Table 2).[40-50] In all studies but one[50] the risk of developing subsequent malignancies was significantly higher in patients with idiopathic VTE than in those with secondary VTE. In the studies in which no extensive screening procedures were performed, the incidence of newly diagnosed malignancy was considerably lower than that observed in studies in which extensive investigation for occult malignancy was performed.[41,42,44,45,47,49] On average, the risk of patients with idiopathic VTE developing a new cancer was four to five times higher than that in patients in whom the thrombotic event was associated with well recognized risk factors.

Besides, two recent articles retrospectively calculated the standardized incidence ratio (SIR) for cancer (the ratio of observed numbers of incident cancers to

Table 1. Incidence (first year) of newly diagnosed malignancy in patients with VTE in comparison to in those without VTE.

Study	*First-year incidence of malignancy*		*OR (95% CI)*
	With VTE (%)	*Without VTE (%)*	*(VTE vs no VTE)*
Gore, 1982[36]	10/133 (8.8)	0/128 (0)	–
Goldberg, 1987[37]	14/370 (3.7)	16/1073 (1.5)	2.6 (1.2-5.7)
Griffin, 1987[38]	4/113 (4.0)	10/517 (2.0)	1.9 (0.4-6.6)
Nordstrom, 1984[39]	66/1383 (4.8)	37/2412 (1.5)	3.2 (2.1-5.0)
ALL			3.2 (2.3-4.5)

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Table 2. Incidence of cancer in the follow-up of patients with idiopathic and secondary VTE.

	Frequency of cancer	
	Idiopathic VTE (%)	*Secondary VTE (%)*
Aderka, 1986[40]	12/35 (34.3)	2/48 (4.2)
Monreal, 1988[41]	3/16 (18.7)	0/67 (0)
Monreal, 1991[42]	7/31 (22.6)	5/82 (6.1)
Prandoni, 1992[43]	11/145 (7.6)	2/105 (1.9)
Monreal, 1993[44]	6/21 (28.6)	3/51 (5.9)
Bastounis, 1996[45]	21/84 (25)	8/202 (4)
Ahmed, 1996[46]	3/113 (2.7)	0/83 (0)
Monreal, 1997[47]	13/105 (12.4)	10/569 (1.8)
Hettiarachchi, 1998[48]	10/155 (6.4)	3/171 (1.7)
Achkar, 1997[49]	13/78 (16.7)	5/154 (3.2)
Rajan, 1998[50]	13/152 (8.6)	8/112 (7.1)
All	112/931 (12.0)	46/1644 (2.8)

those expected) in a large cohort of patients admitted to hospital for VTE. Baron *et al.*, using the Swedish Inpatient Register and the Swedish Cancer Registry, assessed the cancer incidence in 61,998 VTE patients admitted to hospital between 1965 and 1983.[51] Venous thromboembolism was a clear marker of cancer risk, since within the first year after discharge the SIR for cancer was 4.4, and even 10 years later the cancer incidence remained high (SIR, 1.3). Sorensen *et al.*, calculated the SIR in a cohort of 26,610 patients with VTE drawn from the Danish National Registry of Patients for the years 1977-1992.[52] The SIR for cancer was 3.0 during the first 6-month period after discharge, decreased to 2.2 at 1 year and to 1.1 for the remaining period of observation. In both studies the risk of occult cancer was substantial, at least during the first six months after discharge. Furthermore, both studies found strong associations with certain types of cancer (pancreas, ovary, liver and brain).

The extension of DVT might also be considered a risk factor for occult cancer, as suggested by recent data demonstrating that in cancer patients with DVT the contralateral leg is more frequently involved than in patients free from malignancy.[53,54]

Although most studies have indicated a significant association between idiopathic VTE and cancer, the clinical implications of these findings are, as yet, unclear. As suggested by these results, an extensive diagnostic work-up might be justified at the time of referral for the venous thrombosis. Extensive screening with computer tomography scanning, gastrointestinal endoscopy and a number of tumor markers have indeed the potential to detect occult malignancies.[47] However, it remains unclear whether identified malignancies are potentially treatable and whether treatment could favorably influence life expectancy or quality of life. Even if a recent decision analysis of screening for occult cancer in patients with idiopathic VTE revealed potential gains in life expectancy,[55] it should not be forgotten that extensive screening procedures for malignancy are associated with high costs, and themselves carry some morbidity, thus they are only acceptable if life-saving.[56] A clinical trial in which patients with unexplained thrombosis, but asymptomatic for malignant disease are randomized to either extensive screening or standard clinical care without screening, is currently in progress, and has the potential to identify the effect of screening for malignancy on the survival of these patients.[57] In the mean time, clinical decisions must be based on indirect evidence. While waiting for the results of this trial, it is appropriate to maintain a low threshold of suspicion for malignancy when treating patients with unexplained VTE. Decisions to perform additional diagnostic tests can be based on the findings of an initial clinical evaluation, which includes medical history, physical examination, routine laboratory tests and chest X-ray. This approach has received recent support from the retrospective analysis of a wide cohort of patients with idiopathic DVT, conducted in the Boston area.[58]

Primary prophylaxis of VTE

Because VTE is often encountered in patients with cancer, some clinicians have proposed that all patients with cancer should receive pharmacological prophylaxis.[59] However, further trials are needed before this approach can be endorsed.

Currently, primary prevention should be considered for cancer patients in certain circumstances, such as after surgical interventions, during chemotherapy, and in those with indwelling central venous catheters.

Surgical interventions

Patients with cancer are at a markedly high risk of developing DVT. As shown in Table 3, the overall incidence of postoperative DVT in patients with cancer is about twice as high as that of patients free of malignancy.[3-6,60-62] As recently demonstrated by Huber *et al.*, the incidence of post-operative pulmonary embolism is remarkably higher in patients with cancer than in those without cancer.[63]

In order to reduce the risk of venous thrombosis, a Consensus Statement has recently recommended the use of low-dose, low molecular weight heparin (LMWH) or physical measures in patients with cancer when confined to bed for any reason, and when undergoing low-risk surgical procedures.[64] Extensive abdominal or pelvic surgery places patients with cancer at a remarkably high risk of developing post-operative DVT and pulmonary embolism. These patients, therefore, require prophylactic measures comparable to those usually recommended for major orthopedic surgery. These measures include adjusted-dose heparin, higher doses of heparin fractions (on average twice as high as those suggested for general surgery), or oral anticoagulants.[64]

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Table 3. Post-operative DVT following general surgery in patients with or without cancer.

	Cancer patients	*Non-cancer patients*
Kakkar, 1970[3]	24/59 (41%)	38/144 (26%)
Hills, 1972[4]	8/16 (50%)	7/34 (21%)
Walsh, 1974[5]	16/45 (35%)	22/217 (10%)
Rosemberg, 1975[60]	28/66 (42%)	29/128 (23%)
Sue-Ling, 1986[6]	12/23 (52%)	16/62 (26%)
Allan, 1983[61]	31/100 (31%)	21/100 (21%)
Multicenter Trial, 1984[62]	9/37 (22%)	13/53 (24%)
All	128/346 (37%)	146/738 (20%)

As compared to the standard heparin regimen that is used in the prevention of thromboembolism in patients with cancer who undergo surgery, no selective advantage has yet been shown with LMWHs.[65] In a recent double-blind multicenter trial addressing the value of enoxaparin for prevention of DVT in elective cancer surgery, 1,115 patients were randomized to receive either enoxaparin, 40 mg once daily beginning 2 h before surgery, or unfractionated low-dose heparin, 5,000 U three times daily.[66] Primary outcome was VTE as detected by mandatory bilateral venography or pulmonary scintigraphy. Venograms were inadequate in about 40% of patients. Of 631 evaluable patients, a total of 104 (16.5%) developed thromboembolic complications. The frequency was 18.2% in the heparin group and 14.7% in the enoxaparin group. There were no differences in bleeding events or other complications, nor were there differences in mortality at either 30 days or 3 months. Another study compared two doses of a LMWH (dalteparin, 5000 or 2500 units once daily) for thromboprophylaxis in 2070 patients undergoing elective general surgery for abdominal diseases, 63% of whom had malignant disease.[67] The higher dosage schedule reduced the incidence of DVT from 12.6 to 6.7% at the expense of more hemorrhagic complications (4.7 versus 2.7%). This higher rate of bleeding was not seen among patients undergoing operations for cancer.

In this context glycosaminoglycans show promise. Danaparoid (a mixture of dermatan and heparan sulphate) has recently been shown to be as effective and safe as standard heparin for prevention of DVT after elective surgery for malignant disease.[68] Finally, in a recent Italian multicenter trial addressing the value of dermatan sulphate for prevention of DVT in elective cancer surgery, 842 patients were randomized to receive either dermatan sulphate, 300 mg once daily, starting on the second day before surgery, or unfractionated low-dose heparin, 5000 U three times daily.[69] Primary outcome was DVT, as assessed by bilateral contrast venography at the end of treatment. Adequate venography was obtained in 521 patients. Total DVT rate was 40/267 (15.0%) with dermatan sulphate and 56/254 (22.0%) with heparin (p=0.03). The rate of bleeding complications was acceptably low in both groups of patients.

Chemotherapy

As shown in Table 4, patients with breast cancer are at a particularly high risk of developing both venous and arterial thromboses when they receive chemotherapeutic drugs.[70-78] Moreover, a recent trial randomized a large series of women with breast cancer to receive either tamoxifen alone or in association with a 6-month course of chemotherapy.[79] During the study period, thromboembolic events were observed among women allocated to receive the chemotherapy much more frequently than in women allocated to tamoxifen alone. The thrombotic risk of cancer patients receiving chemotherapy is probably increased by the use of hematopoietic colony-stimulating factors.[80] Thromboembolism related to chemotherapy represents, therefore, a relatively common and serious complication of chemotherapy in cancer patients. This risk should be considered when assessing an adjuvant chemotherapy program.

Recently, a prospective double-blind randomized study showed that during chemotherapy very low-dose warfarin (1 mg/day) for six weeks, followed by doses that maintained the International Normalized Ratio (INR) at 1.3 to 1.9, was an effective and safe method for prevention of thromboembolism in patients with metastatic breast cancer.[81] Based on data from this trial, a cost-effectiveness analysis was concluded, showing that warfarin at low doses can be given to women with metastatic breast cancer receiving chemotherapy with no increase in health care costs.[82] Whether this strategy may also be utilized in patients with other oncologic patterns remains to be demonstrated.

Table 4. Arterial and venous thromboses in patients with breast cancer undergoing chemotherapy.

Author	*No. of patients*	*Stage*	*Thrombosis (%)*	*Type of thrombosis*
Weiss, 1981[70]	433	II	5	V
Goodnough, 1984[71]	159	IV	15	V+A
Levine, 1988[72]	205	II	7	V+A
Wall, 1989[73]	1014	Various	1.3	A
Fisher, 1989[74]	383	II	3	V
Saphner, 1991[75]	2352	Various	5	V+A
Clahsen, 1994[76]	1292	Various	2	V
Rifkin, 1994[77]	603	II	2.5	V+A
Pritchard, 1996[79]	353	II	9.6	V+A
Tempelhoff, 1996[78]	50	II	10	V

V = venous thrombosis; A = arterial thrombosis.

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Central venous catheters

Following the demonstration that a strong association exists between the insertion of central venous catheters and the occurrence of upper extremity deep vein thrombosis (UEDVT),[83] a few studies using venography demonstrated that patients with cancer are at a particularly high risk of this complication.[84,85]

Two randomized, controlled studies have documented the benefit of a low-dose of warfarin sodium in decreasing the incidence of thrombosis related to indwelling central venous catheters.[85,86] Subcutaneous administration of LMWH (dalteparin) at the dosage of 2500 IU once daily for 90 days was also proven to be highly beneficial in the prevention of UEDVT in cancer patients with venous access devices.[87]

Treatment and secondary prophylaxis of VTE

Patients with cancer developing an acute thromboembolic disorder should receive a proper course of full-dose unfractionated heparin, i.e., a heparin regimen that prolongs the APTT to 1.5-2.5 times the control value.[12] Alternatively, therapeutic doses (adjusted to body weight) of a low molecular weight heparin (LMWH) can be employed. Thrombolytic drugs are rarely indicated. The limited cases in which thrombolysis may be considered include massive pulmonary embolism, extension of venous thrombosis despite extensive anticoagulation, and upper extremity thrombosis in patients who have an indwelling central venous catheter, which must be kept patent.[24]

Whenever possible, heparin should be administered as soon as there is a reasonable possibility that venous thrombosis exists. Heparin should be overlapped and followed by an oral anticoagulant drug.[12,24]

What are the main questions clinicians confront when facing cancer patients with an episode of venous thrombosis? The main controversies concern the most appropriate duration and intensity of anticoagulation; the risk of extension and/or recurrence of venous thromboembolism during anticoagulation; and the potential for an increased risk of bleeding during the course of proper anticoagulant therapy.

Duration and intensity of anticoagulation

It is a common experience that patients with active cancer remain at a high risk of developing thromboembolism after discontinuation of warfarin therapy.[12,24] In a recent prospective cohort study assessing the long-term follow-up of a large cohort of outpatients with acute DVT, the risk ratio of developing both early and late symptomatic VTE recurrences in cancer patients was 1.74.[88] This means that, after suffering an episode of DVT, cancer patients have a risk of recurrences which is almost twice as high as that observed in patients free from malignancies. In view of the persistently high risk for recurrent thrombotic events and the acceptable risk of bleeding, prolongation of warfarin should be considered for as long as the cancer is active. The suggested policy is to administer warfarin to maintain the INR between 2.0 and 3.0.[12,24]

Recurrence of venous thromboembolism during oral anticoagulation

The literature contains many reports of persistent or recurrent thrombosis in cancer patients despite administration of therapeutic doses of oral anticoagulants. However, the exact frequency of these failures is unknown.

Recently, we reported the data from the long-term follow-up of 823 consecutive patients with DVT.[88-90] All patients received oral anticoagulation for at least three months. Overall the frequency of thromboembolic recurrences during the first three months of anticoagulation was significantly higher in patients with cancer (Table 5). These findings have been confirmed by a multicenter trial addressing the value of LMWH for the initial treatment of acute VTE.[91] More than 1000 patients with VTE were randomized to receive either fixed-doses LMWH or adjusted-dose unfractionated heparin. Irrespective of the study treatment, among the 232 patients with cancer at baseline, 20 (8.6%) had symptomatic recurrent VTE during the 3-month follow-up, as compared to only 32 (4.1%) of the remaining 789 patients ($p<0.001$). Proper studies are required to identify more effective therapeutic approaches in cancer patients suffering an episode of VTE.

The anticoagulation strategy in the treatment of patients with recurrent venous thromboembolism during oral anticoagulation is not rigidly standardized.[12,24] Our policy is to administer a new course of full-dose unfractionated or low molecular weight heparin, followed by a higher dose of warfarin (such as to keep the INR between 3.0 and 4.5). We recommend the use of subcutaneous heparin in adjusted doses for patients who are resistant even to high doses of warfarin. In patients with a very poor prognosis, it seems reasonable to replace warfarin with heparin, without waiting for the eventual failure of higher doses of warfarin. If heparin therapy fails, the only option remains the insertion of a vena cava filter.

Table 5. Venous thromboembolism and bleeding complications during 3 months of oral anticoagulation. A prospective cohort study in 823 consecutive patients with DVT treated with heparin followed by warfarin (experience of the Padua center between 1985 and 1997).

	Cancer	*No cancer (n=189)*	*p value (n=634)*
VTE recurrence	27 (14.3%)	24 (3.8%)	<0.001
Total bleeding	22 (11.6%)	47 (7.4%)	> 0.2
Major bleeding	9 (4.8%)	18 (2.8%)	> 0.2

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Hemorrhagic risk related to anticoagulation

It is generally agreed that cancer patients are at high risk of hemorrhagic complications while receiving oral anticoagulant drugs.[92] However, in our cohort of patients with DVT the risk of bleeding during oral anticoagulation was not different in patients with cancer than in those without cancer (Table 5).[88-90] This finding is supported by a recent study.[93] Bona *et al.* prospectively followed a large number of patients with and without cancer who required long-term anticoagulation. They did not find appreciable differences between the two groups in terms of hemorrhagic complications. The practical implication of these studies is that, at least in the absence of contraindications, there is no need to reduce the intensity of anticoagulation in cancer patients, as is often done in many centers, because of the fear of hemorrhagic complications. It is important to stress that a hemorrhagic complication of the gastrointestinal or genitourinary tract in a patient on oral anticoagulants within the range can be considered a hint in the direction of a hidden cancer.[94]

Reduction of mortality

Anticoagulant treatment of cancer patients, particularly those with lung cancer, has been reported to improve survival.[95,96] These interesting, although preliminary, results of controlled trials lent some support to the argument that activation of blood coagulation plays a role in the natural history of tumor growth.

Numerous studies have been performed in recent years that have addressed the value of LMWH in comparison to standard heparin in the treatment of venous thromboembolism, and an updated meta-analysis of the most adequate reports was published in 1997.[97] In eight of the nine studies reporting on the long-term follow-up (three to six months) of enrolled patients, the analysis of total mortality exhibited a surprising trend in favor of LMWH (pooled relative risk, 0.74; 95% CI, 0.57-0.97).[97] In the five studies that provided subgroup analyses, this effect was entirely attributable to the differences in the subgroup of patients with cancer (Table 6).[91,98-101] This difference cannot be solely attributed to thrombotic or bleeding events. Since large numbers of cancer patients were included in the studies, it seems unlikely that those with more advanced tumors were present in the standard heparin group. While it is also possible that standard heparin increases cancer mortality, such an adverse effect has not been reported previously. These considerations suggest that LMWH might exert an inhibitory effect on tumor growth that is not apparent with standard heparin.[12,102]

The evidence of lowered cancer mortality in patients on LMWH has stimulated renewed interest in these agents as antineoplastic drugs. A few multicenter studies aimed at investigating this fascinating hypothesis are now being carried out.

Table 6. Cancer-related mortality in patients with proximal vein thrombosis. Analysis of prospective randomized trials comparing standard heparin with LMWH treatment.

Series	*UFH no. (%)*	*LMWH no. (%)*	*p value*
Prandoni, 1992[98]	8/18 (44.4)	1/15 (6.7)	
Hull, 1992[99]	13/49 (26.5)	6/47 (12.8)	
Koopman, 1996[100]	7/36 (19.4)	9/34 (26.4)	
Levine, 1996[101]	13/57 (22.8)	8/46 (17.4)	
Columbus, 1997[91]	27/113 (23.9)	20/119 (16.8)	
All	68/273 (24.9)	44/261 (16.8%)	0.03

UFH = unfractionated heparin; LMWH = low-molecular-weight heparin.

Conclusions

Patients with otherwise unexplained VTE have a relatively high risk of subsequent malignant disease. Although extensive screening at the time of a patient's referral has the potential to detect occult malignancies, the cost-to-benefit ratio of an extensive diagnostic work-up still has to be demonstrated definitively.

During prolonged immobilization for any reason, and following surgical interventions, patients with cancer are at a remarkably higher risk of VTE than patients free from malignant disorders. Unfractionated heparin in adjusted doses or LMWH in doses commonly recommended for high risk surgical patients is the prophylactic treatment of choice for cancer patients undergoing an extensive abdominal or pelvic intervention. Furthermore, the risk of thrombotic episodes is increased in cancer patients by chemotherapy and by the use of indwelling central venous catheters. Recent data suggest a positive benefit-to-risk ratio of the systematic use of fixed minidoses of warfarin or low doses of a LMWH.

After experiencing an episode of thrombosis, cancer patients remain at risk of recurrence for as long as the cancer is active. They should, therefore, be protected by a long-term course of oral anticoagulation. The risk of recurrent thrombotic events despite adequate anticoagulation is higher in patients with advanced cancer. Subcutaneous heparin therapy should be reserved for patients in whom warfarin has been ineffective.

Finally, in cancer patients affected by DVT, treatment with LMWHs has been reported to be associated with a lower mortality than treatment with unfractionated heparin therapy. This observation suggests that these agents might have an antineoplastic activity.

Contributions and Acknowledgments

PP planned the review and was responsible for writing the paper. AP critically read all potentially helpful articles and identified those suitable for reviewing. AG critically revised the manuscript for important intellectual content. All authors read the manuscript and approved its final version.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received November 6, 1998; accepted February 3, 1999.

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Haematologica 1999; 84:446-451 molecular basis of disease

Lupus anticoagulants, thrombosis and the protein C system

NAOMI L. ESMON,*§ MIKHAIL D. SMIRNOV,* OMID SAFA,* CHARLES T. ESMON*§‡°

*Oklahoma Medical Research Foundation; §Departments of Pathology, and °Biochemistry & Molecular Biology, University of Oklahoma Health Sciences Center; and ‡Howard Hughes Medical Institute, Oklahoma City, OK, USA

ABSTRACT

Although lupus anticoagulants (LAs) are immunoglobulins that inhibit procoagulant reactions *in vitro*, these molecules are associated with thrombosis *in vivo*. We and others have hypothesized that this may be due to selective targeting of the activated protein C (APC) anticoagulant pathway. Populations of antibodies that interact with protein C or protein S in ways that inhibit their activity are obvious candidates for such pathological molecules. However, it is less clear how populations that appear to bind to membrane surfaces might target the APC anticoagulant complex selectively. Studies now show that the membrane requirements of the APC anticoagulant complex are significantly different from those of the procoagulant reactions. The most dramatic difference is the requirement for the presence of phosphatidylethanolamine (PE) in the membrane for optimal APC function. The inhibitory activity of at least some LAs is enhanced by the presence of PE, but the anti-APC activity is enhanced even more, resulting in the plasma from these patients clotting faster than normal when APC is present. Structure-function studies have been undertaken to understand the PE dependence of this reaction better. Chimeric proteins in which all or part of the Gla domain of protein C has been replaced by the homologous region of prothrombin have been prepared. Unexpectedly, the PE dependence resides primarily in the C-terminal half of the Gla domain. Using liposomes of various composition, we found both the presence of the PE head group and unsaturation of the fatty acid chains are required for optimal inactivation of factor Va. It is hoped that a better understanding of the biochemistry of these reactions, combined with the use of the chimeric proteins described, will permit us to design better assays for the identification of pathologic LAs.

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Key words: lupus anticoagulant, thrombosis, protein C system

Lupus anticoagulants (LAs), although defined according to their ability to block the phospholipid based coagulation cascade *in vitro*, are associated with thrombosis *in vivo*. Many diverse mechanisms have been proposed to account for this apparent dichotomy.[1-3]. Based on what is known about the central importance played by the protein C anticoagulant pathway,[4] we and others[5-7] have proposed that selective inhibition of this system is a logical mechanism by which at least a population of LAs might be prothrombotic. It is readily apparent how inhibitory antibodies specific for the proteins involved would be prothrombotic. Some of these antibodies might be directed to neoepitopes that are expressed on the proteins only in the presence of phospholipid. However, it is less apparent how antibodies which show LA activity in an assay in which activated protein C (APC) is not functional (such as those used to define LA activity) or show reactivity with membrane or phospholipid surface would lead to hypercoaguability. In order to understand how selective inhibition might occur, it is necessary to understand the structure/function relationships of the anticoagulant reactions relative to those of the procoagulant reactions.

Membrane requirements of the coagulation complexes and lupus anticoagulants

For many years, it was believed that the membrane requirements for all of the coagulation reaction complexes were the same. That is, they all required negatively charged phospholipids and phosphatidylserine (PS) was the preferred phospholipid for these membranes.[8] However, this was mostly a self-fulfilling prophecy, as the majority of the studies were performed with the prothrombinase complex and then generalized to the other complexes of interest. In addition, some of the molecules of potential interest, such as phosphatidylethanolamine (PE) do not behave ideally, making some of the studies difficult. This is of some importance, as PE has been implicated as part of the antigenic specificity of clinically relevant LAs.[9-12] We therefore asked two basic questions. First, are the membrane requirements of the protein C anticoagulant pathway really the same as those for the procoagulant complexes? Secondly, if they are not the same, do the characteristics of the anticoagulantly active membranes more closely mimic those of prothrombotic LAs?

PE was added to liposomes containing 20% PS with the remainder made up with phosphatidylcholine (PC). The ability of these vesicles to support prothrombin activation or factor Va inactivation by activated protein C was determined using purified com-

Correspondence: Naomi L. Esmon, Ph.D., Oklahoma Medical Research Institute, Cardiovascular Biology, Research Program, 825 N.E.13th Street, Oklahoma City, Oklahoma 73104, USA. Phone: international +1-405-271-7399 – Fax: international +1-405-271-3137.

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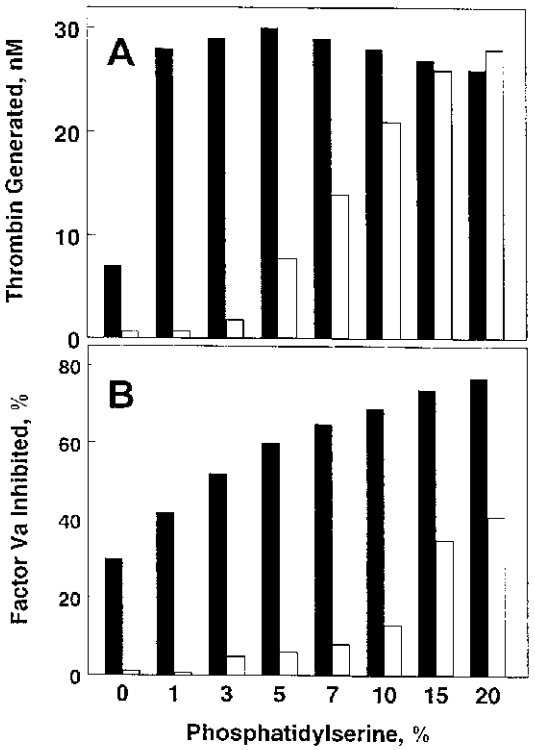

Figure 1. The dependence of the prothrombinase and the APC complex activities on the phosphatidylserine content of vesicles containing 50% phosphatidylethanolamine. A. Prothrombin was activated using vesicles containing the mole % PS indicated in the presence (solid bars) or absence (open bars) of 50% PE. Reaction conditions were: 1.4 µM prothrombin, 0.2 nM factor Va, 2 nM factor Xa and 14 µg/mL phospholipid. B. Factor Va was inactivated essentially as described[13] using vesicles containing the mole % PS indicated in the presence (solid bars) or absence (open bars) of 50% PE. Reaction conditions were: 0.2 nM factor Va, 4 pM APC, 70 nM protein S, 20 µg/mL phospholipid. Residual Va activity was measured in a prothrombinase assay as in part A after inhibition of the APC using 2 nM factor Xa, 1.4 µM prothrombin, 20 µg/mL 20%PS:PC vesicles. Thrombin generated in both cases was determined using a chromogenic assay. (*From Esmon NL. Thrombogenic mechanisms of antiphospholipid antibodies. Thromb Haemost 1997; 78:79-82. ©1997 F.K.Schattauer Verlagsgesellschaft mbH.*)

ponents.[13,14] A dramatic difference was observed. Although the rate of prothrombin activation was only slightly affected by the addition of PE, the ability of APC to inactivate factor Va was essentially dependent upon the presence of PE in the vesicles. In contrast, the addition of cholesterol to these liposomes, which would affect overall fluidity, greatly inhibited the inactivation of factor Va.[14] Others have observed negligible effects of the addition of cholesterol to similar vesicles on prothrombin activation.[15] The degree of factor Va inhibition was linearly dependent on the amount of PE present up to the maximum tested, 40%.[14] Vesicles containing 40% PE were also tested for their activity in plasma clotting assays by a modification of the dilute Russell's viper venom assay.[5] As was observed with purified components, although these vesicles enhanced the clotting time in the absence of APC somewhat, the ability of APC to anticoagulate the plasma effectively was dependent on the presence of PE.[5,14]

The presence of PE in the vesicles also changed how much PS was necessary for optimal activity in the two reactions (Figure 1). When PE was present at 50%, 1% PS was sufficient for the maximal rate of prothrombin activation observed with 20% PS in the absence of PE. However, the maximal rate obtained was unaltered.[16] This is in general agreement with other researchers' results.[15] PE has also been found to have similar effects on other procoagulant reactions.[17,18] In the case of APC activity, however, although the presence of PE decreased the amount of PS required for maximal activity, the effect was not as dramatic as that observed for prothrombin activation. More importantly, there was no concentration of PS that resulted in an equivalent rate of factor Va inactivation as that observed when both PE and PS were present in the membranes.

The presence of PE in the membrane affects the structure beyond the mere presence of a different head group. PE is known to induce the formation of hexagonal phase II structures in the lipid bilayer and some have reported that it is this hexagonal phase II structure of the membrane which is required for binding of LAs.[19] The different degrees of saturation present in the PE component used here and by others also affects membrane fluidity and/or the hexagonal phase-forming properties of the PE. In the case of prothrombin activation, once some degree of fluidity is present[20] due to the presence of unsaturated fatty acids on the phospholipids or the presence of cholesterol, additional fluidity or promotion of the hexagonal phase is not required for enhanced activation.[15,21] Gilbert and Arena also concluded that the enhanced factor VIII binding they observed when PE was present was not related to the hexagonal phase properties of PE.[18] It was therefore of interest to know whether the PE effects observed in the case of APC function were due to the PE head group *per se*, or to the greater degree of fatty acid unsaturation present in this phospholipid.

When the traditional palmitoyl (C16:0) oleoyl (C18:1) phosphatidyl choline (POPC) was replaced with dilinoleoyl (diL; C18:2 in both fatty acid positions) PC, there was a significant increase in prothrombin activation (Figure 2) consistent with that which has been observed previously.[21] Addition of POPS had the expected effect. The addition of PE, no matter what its degree of unsaturation (dipalmitoyl-PE (diPPE) being fully saturated), had no additional enhancing effect. If anything, the presence of PE inhibited the rate of prothrombin activation. The situation is very different in the case of APC inactivation of fac-

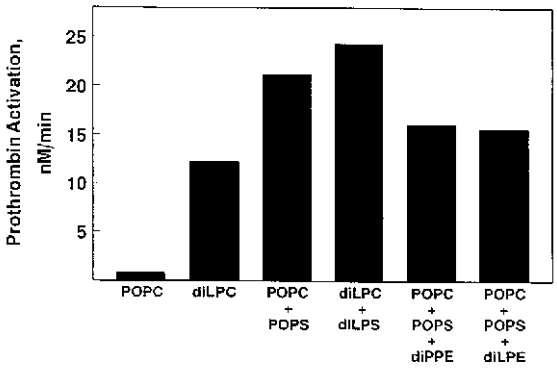

Figure 2. Prothrombinase does not require the PE head group or polyunsaturation for optimal activity. Prothrombin was activated using 10 µg/mL phospholipid of the compositions indicated. When present, PS was at 20% and PE was at 40%. Reaction conditions were: 0.2 nM factor Va, 2.0 nM factor Xa and 1.4 µM prothrombin. *Abbreviations: PO, palmitoyl, oleoyl-; diL, dilinoleoyl-; diP, dipalmitoyl-.*

tor Va. As expected, the addition of POPS to POPC had very little effect (Figure 3, solid bars). Addition of fully saturated diPPE greatly increased the rate of inactivation, but the polyunsaturated form, diLPE, was required for maximal stimulation. As can be seen on the right side of the figure, just the presence of the diunsaturated diLPC:diLPS improved inactivation a small amount, possibly indicating increased fluidity alone has some enhancing effect. Again, the addition of diPPE had a significant effect but the presence of some degree of unsaturation in the PE moiety was required for maximal effect. We would conclude that both the PE head group *per se* and polyunsaturation in some membrane component is required for optimal inactivation of factor Va by APC. Although both features can affect the observed rate of prothrombin activation, neither is required for optimal activity. Similarly, although the presence of the PE head group and/or polyunsaturation or other molecules that alter membrane fluidity can affect the activity of other procoagulant reactions, neither is required for achieving near optimal activity.

As we have shown previously, PE is required to observe the anti-APC activity of a variety of LA plasmas.[5] PE also enhances LA activity in the absence of APC. However, when purified reagents were employed, no conditions were found in which the prothrombinase reaction could be inhibited more than 50% by this particular immunoglobulin studied in detail.[14] This is probably not sufficient inhibition for an individual to be effectively "anticoagulated" by the antibody.[22] In contrast, the APC activity could be inhibited >90%, thus leading to a potential overall hypercoagulable state. Interestingly, the titer of this immunoglobulin was improved by inclusion of more PS in the liposomes (manuscript in preparation), just as the activity of the APC complex was improved by this inclusion (Figure 1). Thus, not only are the membrane requirements of the anticoagulant complex different from those of the procoagulant complexes, these requirements are also similar to those of at least a population of LAs associated with thrombosis and may be the basis for a link between the protein C pathway, lupus anticoagulants and thrombosis.

Structure-function studies of protein C: what is important for LA activity?

In order to understand the protein C-phospholipid interaction better, protein structure studies were undertaken. It was hoped that these studies might also lead to a better understanding of the LA inhibitory activity and possibly to useful reagents. Protein C is a vitamin K dependent protein, and contains the amino terminal Gla domain characteristic of such

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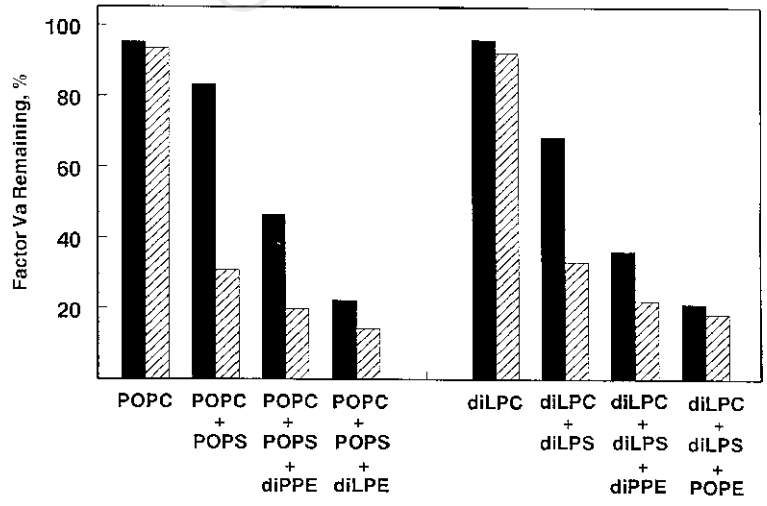

Figure 3. Both the PE head group and polyunsaturation are required for optimal APC activity of native APC but not the APC-Pt Gla chimera. Factor Va was inactivated essentially as described[24] using 10 µg/mL phospholipid of the compositions indicated. When present, PS was at 20% and PE was at 40%. Abbreviations are defined in the legend to Figure 2. Reaction conditions were: 0.2 nM factor Va, 5 pM APC (solid bars) or APC-Pt Gla (hatched bars).

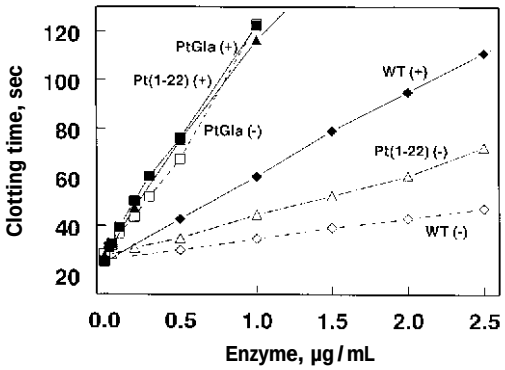

Figure 4. The effect of PE in liposomes on the anticoagulant activity of native or chimeric APC. Vesicles containing 20% PS, 80% PC (–) at 24 µg/mL or 40% PE, 20% PS, 40% PC (+) at 8 µg/mL were used in a one-stage clotting assay in the presence of APC, APC-Pt Gla or APC-Pt(1-22). Different phospholipid concentrations were necessary for the starting clotting times in the absence of APC to be similar. Mixtures contained 2.5 ng/mL factor X-activating enzyme from Russell's viper venom and a standard human plasma pool. Clotting was initiated by the addition of $CaCl_2$. (*From Smirnov MD. A Chimeric Protein C Containing the Prothrombin Gla Domain Exhibits Increased Anticoagulant Activity and Altered Phospholipid Specificity. J Biol Chem 1998: in press. Copyright © 1998 the American Society for Biochemistry and Molecular Biology, Inc.)*

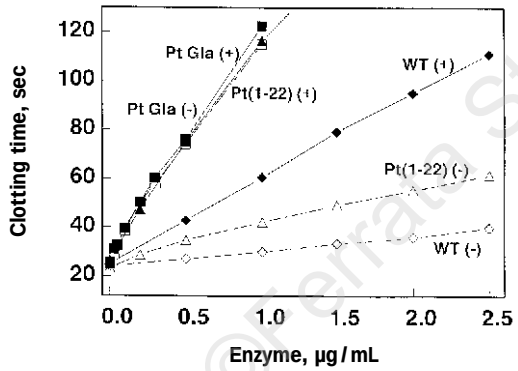

Figure 5. The effect of protein S on the anticoagulant activity of native or chimeric APC. Plasma clotting was initiated as described in Figure 4 using 8 µg/mL vesicles containing 40% PE, 20% PS, 40% PC in the presence or absence of an inhibitory monoclonal antibody to protein S (S155, 300 µg/mL). (–), no protein S activity present; (+) protein S activity present. (*From Smirnov MD. A Chimeric Protein C Containing the Prothrombin Gla Domain Exhibits Increased Anticoagulant Activity and Altered Phospholipid Specificity. J Biol Chem 1998; 273:9031-40. ©1998 the American Society for Biochemistry and Molecular Biology, Inc.*)

proteins.[23] This highly homologous domain has been implicated in membrane binding. It seemed likely that the small differences within this region between protein C and prothrombin might be responsible for the differences in the PE dependent behavior. Two chimeras were therefore constructed; one in which the entire Gla domain and hydrophobic stack of protein C was replaced with that of prothrombin, called PC-Pt Gla, and a second in which only residues 1-22 representing the amino terminal half of this domain was swapped, called PC-Pt(1-22). The properties of these mutants were compared with those of the wild type protein.[24] As predicted, the activated PC-Pt Gla (APC-Pt Gla) was no longer significantly influenced by the presence of PE in the liposomes (Figure 4). This mutant was also only slightly influenced by the fatty acid composition of the phospholipids used (Figure 3, hatched bars). However, there were two other, unexpected results. First, the APC-Pt(1-22) protein retained the sensitivity to the presence of PE in the membrane, indicating that it is the C-terminal half of the Gla domain that is responsive to this property of the membrane. Most studies have focused on the amino terminal of the Gla domain[25] for insights into the protein-membrane interactions of this class of proteins. However, examination of the structure of this region as modeled from the crystal structures of prothrombin[26] and factor VII[27] indicates how this region may be involved in membrane surface interaction (see ref. #24 for discussion). The second unexpected result was the significantly greater anticoagulant activity of both of the chimeric proteins. This property is not fully understood. Preliminary results suggest that prothrombin can inhibit the activity of the wild type protein more potently than the APC-Pt Gla chimera.[28] Other factors that may be inhibiting the native protein more than the chimera are not known, but these chimeras may be useful probes to identify these putative inhibitors. These properties of the PC-Pt Gla chimera may be very useful both clinically and diagnostically. Clinically, the chimera may be useful as an anticoagulant in patients with strong PE dependent, prothrombotic LAs. Diagnostically, it may be a useful reagent for the identification of PE dependent LAs that inhibit the APC complex.

These chimeras were also used to investigate the interaction of APC with its cofactor, protein S, in factor Va inactivation. These studies revealed yet another interesting feature about protein C and a potential use for the chimera. As can be seen in Figure 5, not only is the APC-Pt Gla insensitive to the presence of PE in the liposomes, it is also insensitive to the presence of protein S in the plasma. In contrast, the APC-Pt(1-22) retains protein S dependence. Again this indicates that it is the C-terminal region of the Gla domain of protein C that is required for proper interaction with another component of the complex. The APC-Pt Gla should also be a useful therapeutic agent in patients with protein S deficiency, whether chronic or acute due to an inflammatory state.[29] This would include those patients who are protein S deficient due to anti-protein S antibodies, a situation observed in a significant number of LA patients with thrombosis.[6,30-32]

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Summary

In conclusion, the membrane requirements for the activated protein C anticoagulant complex differ significantly from those of the procoagulant complexes. These properties, in particular the requirement for PE in the membrane, mimic the lipid requirements for at least a population of LAs associated with thrombosis. In addition, in studies designed solely to investigate the basic biochemistry of the molecules involved, a reagent was developed which may have significant diagnostic and clinical potential.

A most satisfying result indeed.

Contributions and Acknowledgments

All the authors contributed to design the study.

Funding

The research discussed herein was funded by a grant awarded by the National Heart, Lung and Blood Institute of the National Institutes of Health (No. P50 HL54502).

Disclosures

Conflict of interest: none

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received November 6, 1998; accepted January 15, 1999.

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Haematologica 1999; 84:452-460 trends in hematology

Antithrombin replacement in patients with sepsis and septic shock

DANIELA GIUDICI,* FRANCESCO BAUDO,# GUALTIERO PALARETI,° ADRIANO RAVIZZA,^ LORENZA RIDOLFI,@ ARMANDO D'ANGELO§

**Unità di Terapia Intensiva e §Servizio di Coagulazione, IRCCS H S.Raffaele, Milan; #Unità di Emostasi e Trombosi e ^Dipartimento di Terapia Intensiva, Ospedale Niguarda, Milan; °Dipartimento di Angiologia e @Unità di Terapia Intensiva, Ospedale S. Orsola, Bologna, Italy*

ABSTRACT

Sepsis is a frequent complication of critically ill patients and its incidence is increasing. Currently, septic shock is the most common cause of death in non-coronary intensive care units. Over the last 10 to 15 years, new antibiotics and increasingly sophisticated critical care have had little impact on the mortality rate of septic shock. The italian SESPIS Study, carried out in 99 intensive care units in 1994, reported mortality rates of 52% and 82% for severe sepsis and septic shock respectively. New therapeutic approaches aimed at neutralizing microbial toxins and modulating host mediators have shown some efficacy in large clinical trials and / or in animal models, but to date, no therapy of sepsis aimed at reversing the effects of bacterial toxins or of harmful endogenous mediators of inflammation has gained widespread clinical acceptance. Because of the strong association of severe sepsis with a state of activation of blood coagulation and the potential role of capillary thrombosis in the development of the multiple organ dysfunction syndrome, anticoagulant agents have been tested in the setting of spetic shock. However, neither administration of heparin nor of active site-blocked factor Xa or of anti-tissue factor antibodies has proven effective in preventing deaths due to septic shock in animal models. In contrast, infusion of antithrombin, protein C, or tissue factor pathway inhibitor all resulted in a significant survival advantage in animals receiving lethal doses of *E. Coli*. Antithrombin concentrates have been used in a significant number of critically ill patients. A double-blind, placebo controlled study carried out in 3 Italian intensive care units has recently shown that the administration of antithrombin aimed at normalizing plasma antithrombin activity had a net beneficial effect on 30-day survival of patients requiring respiratory and / or hemodynamic support because of severe sepsis and / or post-surgery complications.
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Key words: sepsis, septic shock, diffuse intravascular fibrin formation, antithrombin replacement therapy, protein C

Correspondence: Armando D'Angelo, M.D., Servizio di Coagulazione, IRCCS H S. Raffaele, via Olgettina 60, 20132 Milan, Italy. Phone: international +39-02-26432228 – Fax: international +39-02-26432640 o E-mail: armando.dangelo@hsr.it

Infection is a common cause of admission into intensive care units (ICUs) and a frequent complication of critically ill patients. A series of factors have contributed to the increasing incidence of sepsis and of septic shock. Immunosuppressive therapy for malignancy, organ transplantation, or inflammatory disease places patients at increased risk of infectious complications. Patients predisposed by underlying diseases such as diabetes mellitus, renal failure, and cancer are more likely to suffer an increased rate of infections because they have now a longer life-expectation. Invasive life support procedures (hemodynamic and respiratory support) and broad-spectrum antibiotics have created a large hospital-based population at risk of nosocomial infection by resistant micro-organisms.

Septic shock is currently the most common cause of death in non-coronary ICU.[1,2] Mortality is related to the severity of sepsis and of the underlying disorder that is nearly always present. Agreement about the definition of a septic syndrome has been only recently achieved. In 1992, a Consensus Conference of the *American College of Chest Physicians and Society of Critical Care Medicine* established a set of definitions that could be applied to patients with sepsis and its sequelae.[3] The term *sepsis* implies a clinical response arising from infection, but a similar, or even identical, response may also develop in the absence of infection. This systemic inflammatory response syndrome (SIRS) can occur following a wide variety of insults, infectious or non-infectious, the latter including pancreatitis, ischemia, multiple trauma and tissue injury, etc. SIRS is defined by the occurrence of two or more of the following conditions: temperature >38°C or <36°C, heart rate >90 beat/min, respiratory rate >20 breath/min or PaCO2 < 32 torr, white blood cell count >12,000/µL or >10% immature forms. When the systemic inflammatory response syndrome is the result of a confirmed infectious process, it is termed sepsis.

Sepsis and its sequelae represent a continuum of clinical and pathophysiological severity. Sepsis is defined as *severe* when it is associated with organ dysfunction, hypoperfusion abnormalities or sepsis-

induced hypotension. Hypoperfusion abnormalities include lactic acidosis, oliguria, or an acute alteration of mental status. Sepsis-induced hypotension is defined by the presence of a systolic blood pressure of <90 mm Hg or its reduction by more than 40 mm Hg from the baseline, in absence of other causes for hypotension (cardiogenic shock etc.). *Septic shock* is a subset of severe sepsis and is defined as sepsis-induced hypotension, persisting despite adequate fluid resuscitation, along with the presence of hypoperfusion abnormalities or organ dysfunction. Patients receiving inotropic or vasopressor agents might no longer be hypotensive by the time they manifest hypoperfusion abnormalities or organ dysfunction, yet they are still considered affected by septic shock.

A frequent complication of SIRS is the development of organ system dysfunction, as a process of progressive failure of several interdependent organ systems. The detection of altered organ function in acutely ill patients constitutes a syndrome that should be termed *multiple organ system dysfunction syndrome* (MODS), in which organ function is not capable of maintaining homeostasis.[4,5] The term *dysfunction* emphasizes the dynamic nature of the process, although specific descriptions of this continuous process are not currently available. Multiple organ dysfunction syndrome is subject to modulation by numerous factors, both interventional and host-related, at varying time periods. MODS may be primary when it occurs early in response to a well-defined insult such as trauma, pulmonary contusion, rhabdomyolysis, massive transfusions, or it may be secondary to the host response to the insult when it is characterized by a generalized activation of the inflammatory reaction in organs remote from the initial insult. When due to infection, secondary MODS usually evolves after a latent period after the provoking injury or event, and is a frequent complication of severe infection.

Pathogenesis

The pathogenesis of sepsis and septic shock are not completely understood. Gram-positive organisms releasing exotoxins, Gram-negative organisms containing endotoxins and fungi can initiate this pathogenic cascade. The process begins with the proliferation of micro-organisms at a nidus of infection. The organisms may invade the bloodstream directly (leading to a positive blood culture) or may proliferate locally and release various substances into the bloodstream. These events trigger host cells (neutrophils, monocyte-macrophages) to release a variety of interacting cytokines (tumor necrosis factor [TNF], interleukins, interferons). This results in the activation of several pathways (complement, coagulation, fibrinolytic, and hormonal) and in the increased production of numerous endogenous mediators (C5a, eicosanoids, endorphins, toxic oxygen radicals, nitric oxide, and platelet-activating factor), with profound physiologic effects on the cardiovascular system and on the function of other organs.[6]

Early in severe sepsis, systemic vascular resistance decreases – primarily mediated by the release of bradykinin and histamine – and cardiac output increases. In this hyperdynamic phase, septic shock is a classic form of distributive shock, resulting from abnormal distribution of blood flow. Despite an often elevated cardiac output, tissue oxygen utilization is reduced.[7,8] The decreased artero-venous oxygen difference suggests that oxygen is not reaching or not being used by tissues. The exact mechanisms responsible for decreased tissue perfusion are poorly understood. In septic shock, many vascular beds are dilated, but some are constricted, and some are occluded by microthrombi. The aggregation of neutrophils and platelets may lead to impairment of blood flow. Neutrophil migration occurs along the vascular endothelium, resulting in the release of many mediators and the migration of neutrophils into tissues. Neutrophils can release active oxygen species, such as superoxide radicals, that can directly damage cells. Components of the complement system, such as C5a, are activated. Inflammatory mediators, such as prostaglandins and leukotrienes are released from many types of cells and can cause either vasoconstriction or vasodilatation, with increased permeability of the vascular endothelium and passage of fluid from the intravascular to the interstitial fluid space. Endothelial damage may *per se* decrease oxygen and substrate utilization by the tissues.

In the hyperdynamic phase blood pressure is normal or slightly reduced, the skin is warm and dry, there is tachycardia, urine output is satisfactory, and the patient hyperventilates and is pyretic. Fever results from the direct effects of endotoxins and interleukin-1 on the hypothalamus. The release of inflammatory mediators and endothelial damage also lead to the development of diffuse intravascular fibrin formation (DIFF) and deposition, followed by a secondary bleeding tendency. DIFF decreases organ blood flow, causing hypoxia, lactic acidosis, organ dysfunction and failure.[9] This occurs especially in the circulation of the lungs, liver, kidneys, and gastric mucosa with the manifestations of secondary MODS.

If the clinical state is not recognized and treated within a few hours the patient enters the hypotensive phase of septic shock, in which the combination of decreased systemic vascular resistance and myocardial depression induces hypotension which is independent from adequate fluid resuscitation.[10] A reversible depression of myocardial function, with decreased ejection fraction and left ventricular dilation, is common in septic shock. Circulating anti-inotropic substances, termed myocardial depressant substances,[11,12] may play an important role in the pathogenesis of myocardial depression. In the hypotensive phase the patient is oliguric with cold, pale skin, and is cyanotic, features which are typical of an established shock syndrome. As a consequence of the arteriolar dilation and of the increased capillary and

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post-capillary venule permeability, especially in the infected tissues, there is increased fluid transfer from capillaries to the interstitial fluid. The hypovolemia decreases venous return, cardiac output and blood pressure. Baroreceptor compensation increases sympathetic activity so causing vasoconstriction in the skin, the splanchnic areas, kidney and muscles.

Bronchoconstriction is an early finding in many patients with severe sepsis. This is probably due to endotoxin or to release of inflammatory mediators. At this time the chest radiograph is often normal, but gas exchange may be mildly abnormal. Later, if septic shock occurs many patients develop diffuse alveolar damage consistent with the *adult respiratory distress syndrome*[13] (ARDS). From 40% to 60% of patients with Gram-negative septic shock develop ARDS.[14] Alveolar-capillary membrane damage allows for leakage of fluid and proteins into the pulmonary interstitium. Alveoli are subsequently flooded, causing a marked increase in intrapulmonary shunting and severe arterial hypoxia. At this stage the chest radiograph demonstrates diffuse bilateral alveolar infiltrates. Hypoxic pulmonary vasoconstriction, *in situ* thrombosis, and aggregation of neutrophils and platelets in the pulmonary microvascular system increase pulmonary artery pressure and right ventricular afterload, leading to a worsening of right ventricular performance.

In addition to the cardiopulmonary systems, other systems may sequentially become dysfunctional in septic shock because of the role of inflammatory mediators. Visceral hypoperfusion and decreased intestinal peristalsis may lead to alterations of the barrier function of the gastrointestinal tract; gastrointestinal bleeding may follow stress ulceration of the gastric mucosa. Liver dysfunction may manifest as hyperbilirubinemia, elevated aminotransferase levels, cholestasis, progressive and intractable hypoglycemia and hypoalbuminemia. As kidney function declines, urine output falls and blood urea and creatinine levels rise. Renal failure is mainly due to acute tubular necrosis induced by hypotension or capillary injury, but drug-induced renal damage may also occur. Alterations of the mental status can occur, ranging from mild confusion and lethargy, to stupor and coma; abnormalities in the blood brain barrier and changes in the concentrations of circulating aminoacids frequently accompany this *obtundation of sepsis*.[15] Abnormalities of the clotting system, ranging from mild prolongation of the prothrombin time and of the partial thromboplastin time, to profound thrombocytopenia and frank disseminated intravascular coagulation are common in patients with septic shock.

Treatment

The treatment strategy for severe sepsis and septic shock is based on the provision of intensive life supports, the eradication of micro-organisms, the neutralization of microbial toxins, and the modulation of host mediators.[16]

Intensive life supports to maintain vital functions involve careful monitoring of patients in a critical care unit setting. Metabolic derangements (electrolyte disturbances, acidosis) should be aggressively corrected, as they can worsen the hemodynamic abnormalities of septic shock. The hematocrit should be maintained above 30% to improve the oxygen-carrying capacity. Respiratory failure requires mechanical ventilation.

Patient monitoring is essential to the choice of the cardiovascular support and includes cardiac rhythm monitoring, intra-arterial invasive blood pressure monitoring, right-sided heart catheterization with a Swan-Ganz catheter, and laboratory monitoring of the metabolic profile.

All patients with severe sepsis and septic shock have moderate to profound intra-vascular hypovolemia due to vasodilatation and loss of fluids in the extravascular spaces. The type and amount of fluid (crystalloids, colloids, and albumin) are highly controversial. When the mean arterial pressure is less than 60 mmHg, volume resuscitation is the initial treatment of choice, to avoid limiting coronary and cerebral artery autoregulation and to prevent inadequate tissue perfusion. Fluids should be infused rapidly to maximize ventricular performance. In general, this can be obtained at a pulmonary capillary wedge pressure of 12 to 15 mm Hg. Patients with higher wedge pressures carry a substantial risk of developing pulmonary edema. If, in spite of volume resuscitation, the mean arterial pressure remains below 60 mmHg when the pulmonary capillary wedge pressure is above 15 mmHg, inotropic agents used singly or in combination may offset the myocardial dysfunction and augment cardiac output. No universal agreement exists as to how these agents should be utilized, in view of their different effects on cardiac stimulation, vasoconstriction and vasodilatation (Table 1). Dopamine is commonly employed in this setting because of the β-adrenergic effects enhancing cardiac performance and the α-adrenergic effects supporting arterial blood pressure. The potent vasoconstrictor effects of norepinephrine are advantageous in septic shock patients who are unresponsive to high doses of dopamine. Dobutamine may be used alone or in combination with other catecholamines to improve cardiac performance.

Eradication of micro-organisms requires early antibiotic administration. This is initially empirical, using broad-spectrum antibiotics against Gram-positive and Gram-negative bacteria and sometimes against fungi. Cultures of body fluids are helpful in the identification of the micro-organisms involved, but radiological investigations may be required to discover the site of infection. Specific foci of infection should be drained and necrotic tissue surgically removed when appropriate.

Septic shock may, however, present with no identifiable source of infection and with negative blood

Table 1. Vasopressor therapy in septic shock.

Inotropic agent	*Cardiac stimulation (β-1)*	*Vaso-constriction (α-1)*	*Vaso-dilatation (β-2)*
Dopamine 5-10 µg/kg/min	++	+	++
↓			
Dopamine 10-20 µg/kg/min	+++	+++	+
↓			
Norepinephrine 0.02-0.2 µg/kg/min	+++	++++	0
+ Dopamine 2-4 µg/kg/min	++	+	++
↓			
Norepinephrine 0.02-0.2 µg/kg/min			
+ Dopamine 2-4 µg/kg/min	++	+	++
+ Dobutamine 5-10 µg/kg/min	++++	+	++

cultures especially in neutropenic patients.

Over the last 10 to 15 years, new antibiotics and increasingly sophisticated critical care have had little impact on the mortality rate of septic shock, which remains extremely high as demonstrated by the results of the Italian SEPSIS Study.[17] The aim of this prospective, multicenter investigation was to evaluate the clinical outcome of consecutive patients admitted to intensive care units on the basis of the diagnostic criteria of the ACCP/SCCM Consensus Conference.[3] The study was carried out in 99 ICUs in Italy from April 1993 to March 1994. In a preliminary analysis of 1100 patients, severe sepsis and septic shock had mortality rates of 52.2% and 81.8% respectively (Table 2). As a result, new therapeutic approaches have been tested, aimed at neutralizing microbial toxins and modulating host mediators (Table 3). Some of the agents have shown some efficacy in large multicenter clinical trials (anti-endotoxin monoclonal antibodies,[18-19]), others only in animal models (monoclonal anti-TNF antibodies,[20-22]). To date however, no therapy of sepsis aimed at reversing the effects of bacterial toxins or of harmful endogenous mediators has gained widespread clinical acceptance.

Antithrombin concentrates in sepsis and septic shock

In view of the strong association of severe sepsis with a state of activation of blood coagulation and the potential role of capillary thrombosis in the development of MODS, anticoagulant agents have been tested in the setting of septic shock. However, neither administration of heparin[23] nor of active site-blocked factor Xa[24] have proven effective in preventing deaths due to septic shock in animal models. Even the administration of anti-tissue factor antibodies did not prevent severe manifestations of septic shock in animal models, although resulting in effective blockade of the clotting system.[25] In contrast, infusion of natural inhibitors of blood coagulation (antithrombin, protein C, tissue factor pathway inhibitor), all

Table 2. Mortality rate of consecutive patients admitted to Italian intensive care units: results of the Italian SEPSIS Study.

ACCP/SCCM diagnosis on admission:		*Nil*	*SIRS*	*Sepsis*	*Severe sepsis*	*Septic shock*
Patients	n.	421	573	50	23	33
	(%)	38.3	52.1	4.5	2.1	3.0
Mortality rate	(%)	24.0	26.5	36.0	52.2	81.8

Table 3. Novel therapeutic approaches in septic shock.

Neutralization of microbial toxins	*Modulation of host mediators*
Anti-endotoxin antibodies:	Anti-TNF antibodies (TNF MoAb)
Polyclonal antibodies (*E.coli* J5 antiserum, antibodies to Lipid A)	Interleukin-1 receptor antagonists
Monoclonal antibodies (HA-1A, E5)	Anti-C5a antibodies
Lipid A analogs (lipid X, monophosphoryl lipid A)	Eicosanoid inhibitors
	Antioxidants
Cationic polypeptide antibiotics	Corticosteroids
	PAF antagonists
Plasma detoxification (plasmapheresis, continuous artero-venous hemofiltration)	Inhibitors of coagulation (antithrombin, protein C)

resulted in a significant survival advantage in animals receiving lethal doses of *E. coli*.[26-29]

Because of their commercial availability, antithrombin concentrates have been used in a significant number of critically ill patients. Antithrombin (AT), a glycoprotein synthesized by the liver and the kidney, is a main physiologic inhibitor of serine proteases generated during blood coagulation (FIIa, FIXa, FXa, FXIa, FXIIa).[30] The rate of neutralization of the above-mentioned proteases is increased by 3 orders of magnitude in the presence of heparin and heparin-sulphate. The concentration of AT in plasma is decreased in conditions associated with diffuse intravascular fibrin formation, particularly in sepsis and shock.[31-34] The decreased plasma concentration of AT may be an indication of the role of DIFF in the pathogenesis of multi-organ failure; it is a poor prognostic factor and correlates with survival.[35-39] Two randomized studies addressed the use of AT concentrate supplementation in the treatment of severe sepsis and shock, but they did not include a placebo-control group. Blauhut *et al.* randomized 51 patients with shock of different etiology (sepsis, trauma, hepatic coma) to receive AT, heparin or AT + heparin.[34] The time to normalization of the platelet count and of the fibrinogen concentration was shorter in patients receiving AT, but no difference in survival was observed. In a subsequent study, including only

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patients with traumatic shock, Vinazzer reported a significant reduction in the mortality of patients treated with AT concentrate.[40] However, in an Italian study of patients with an established diagnosis of DIFF, the administration of AT concentrates did not result in any significant survival advantage.[41] These results are difficult to interpret. In critically ill patients, the evaluation of the efficacy of therapeutic agents requires a double-blind design, to avoid the bias of the attending clinician who is confronted with patients with a potentially fatal outcome.[42] Fourrier *et al.* published the first randomized double blind, placebo-controlled study in septic shock. Patients treated with AT tended to have a survival advantage, but the difference from the placebo group did not reach conventional statistical significance.[43] Similar results were obtained by Lamy *et al.*[44] (Table 4).

We planned a double blind study to evaluate the effect of AT administration on survival of a selected group of patients requiring hemodynamic and/or respiratory support because of severe sepsis and/or post-operative complications.[47] A major assumption was that the observation of decreased AT levels – unrelated to evidence of impaired liver synthesis of the protein – may reflect uncontrolled activation of the clotting system in critically ill patients, with a potentially unfavorable role in their prognosis. In line with this hypothesis, we tested the possibility that the maintenance of normal AT levels by infusion of AT concentrate could have a beneficial effect on survival of critically ill patients irrespective of the causes leading to the requirement for hemodynamic and/or respiratory support.

The study was randomized and double blind, with the inclusion of a placebo control arm. Identification of the infused material by the attending physicians was prevented by the use of identical black bottles, syringes and infusion-sets. Patients were included in the study if they were 18 to 75 years old, were admitted to the intensive care unit (ICU) because of sepsis and/or post-operative complications requiring respiratory and/or hemodynamic support and had plasma AT activity < 70% of normal. Septic shock was defined as sepsis-related hypotension requiring vasoactive drugs for more than 24 hours, persisting despite adequate fluid resuscitation, along with the presence of hypoperfusion abnormalities or organ failure. The respiratory support consisted of assisted or controlled ventilation for more than 24 hours. The hemodynamic support consisted of the administration of inotropic (dopamine or dobutamine, >5 μg/kg/min) and/or vasoactive amines (epinephrine or norepinephrine).

Patients were excluded if they had suffered multiple trauma, had liver cirrhosis or acute liver failure, cancer in terminal phase, immunodeficiency, or leukemia, if they were pregnant, or were being submitted to heparin therapy for hemodialysis, hemofiltration or other indications. Patients receiving heparin prophylaxis were not excluded.

The AT concentrate and the placebo (albumin solution, 50 g/L) were supplied by the manufacturer (Immuno) in identical black bottles containing either 2,000 U of AT or 2 g of albumin in lyophilized form. A fixed dose of 4,000 units of AT or 4 g of albumin were injected as a bolus in 30 min, followed by 1 bottle every 12 hours for 5 days by a pump-driven syringe. There was no limitation to standard medical care in each ICU, but for the infusion of fresh frozen plasma, indicated for patients with active bleeding and/or with PT ratios > 2.0, or of platelet concentrates, which were administered at the dosage of 1 unit/10 kg body weight if the platelet count was $< 50 \times 10^9$/L.

The simplified acute physiologic score (SAPS)[48] was recorded in each patient at admission; a modified multi-organ failure (MOF) score,[47,49] was recorded at admission and daily thereafter for 7 days. Baseline AT determinations for the enrolment of patients were carried out locally in each hospital. Thereafter, no local AT determinations were permitted. AT data reported were obtained after centralized measurement against an established calibrator (Immuno).

The main end-point of the study was survival at 30 days. The sample size was calculated to detect a 50% reduction of the expected mortality in the placebo group (60%) with an α error of 0.05 and a β error of 0.10. This mortality figure was anticipated based on the results of a previous study validating the SAPS score in consecutive patients referred to ICUs.[48] No separate randomization blocks were applied for patients with or without septic shock.

One hundred and twenty consecutive patients were enrolled (60 in each treatment arm) from January 1991 to November 1994 in three ICUs: 92 patients because of post-operative complications, 12 patients because of bronchopneumonia with septic shock, and 16 patients with a miscellany of disorders. One hundred patients had sepsis and 56 had septic shock at admission. The distribution of patients in the two arms was well balanced except for the number of patients with septic shock (33 in the AT arm versus 23 in the placebo arm, p = 0.08) and for the baseline

Table 4. Controlled studies of antithrombin replacement therapy in septic shock and critically ill patients.

Author (ref.)	*No. of patients*	*Mortality rate*		*Odds Ratio*	*95% C.I.*
		Standard treatment	*AT replacement*		
Blauhut *et al.*, 1985[34]	51	12%	15%	1.29	0.18-42.9
GISACID, 1990[41]	41	23%	29%	1.33	0.24-∞
Harper *et al.*,1991[45]	50	32%	32%	1.00	0.26-3.85
Albert *et al.*, 1992[46]	33	31%	25%	0.73	0.11-4.40
Fourrier *et al.*, 1993[43]*	32	50%	28%	0.40	0.06-2.19
Lamy *et al.*, 1996[44]*	42	41%	25%	0.48	0.10-2.16

**Double blind studies.*

MOF score (AT arm: 5.6±2.5; placebo arm 4.8±2.3, p = 0.08). As a result, more patients in the AT group required hemodynamic support (53 in the AT arm versus 42 in the placebo arm, p = 0.04). Forty-nine patients in the AT group and 51 patients in the placebo group had sepsis. The infectious agents were identified in 93 patients by blood, urine and bronchoaspirate cultures and were similarly distributed in the two treatment arms (46 Gram-positive: *S. Aureus, S. Epidermidis*; 44 Gram-negative, *P. Aeruginosa, Serratia, Actinobacter, Enterobacter, K. Pneumoniae, E. Coli*; 34 fungi: *C. Albicans, Aspergillus*; *cytomegalovirus* 1). In patients with septic shock, hemodynamic parameters at entry were similarly abnormal in the two treatment arms.

Some degree of dyshomogeneity between the baseline characteristics of patients enrolled in the three centers participating in the study was observed, with statistically significant differences affecting SAPS (p=0.003), and AT levels (p=0.004), and probably resulted from poor standardization of AT measurements between the 3 laboratories.

Four patients received therapy for less than 24 hours: 1 patient, in the placebo group, was transferred to an another hospital after the bolus infusion and 3 patients (2 in the ATIII group and 1 in the placebo group), included in the intention to treat analysis, died on the day of enrolment. The mean time interval from admission to the ICUs and enrolment into the study (5.0±6.5 days) was not different for patients allocated to AT or placebo. Significant bleeding, requiring transfusion of red blood cell packs and platelet concentrates occurred in 6 patients in the placebo group and 5 patients in the AT group. No differences were observed between the treatment arms with respect to transfusion requirements with fresh frozen plasma, platelet and red blood cell packs. No side effects possibly related to AT treatment were observed.

Changes in plasma AT concentrations were not observed either in patients receiving placebo or in patients receiving AT after the initial rise observed following the first bolus injection (range 98-101%).

Survival curves were calculated for the 119 (intention to treat) and the 116 patients after the exclusion of the early deaths. By Kaplan-Meier analysis survival was not different in the two arms. At day 30, 30 patients in the AT arm (50%) and 27 patients in the placebo arm (46%) were alive. Because of the unbalanced randomization for baseline variables potentially affecting survival, we analyzed, by the Kaplan-Meier approach, the influence on survival of the requirement for hemodynamic support, the presence of septic shock and the MOF score at entry. The presence of septic shock (p<0.0001) and the requirement for hemodynamic support (p<0.0001) were negatively associated with survival; 30-day mortality was 75% in patients with septic shock and 32% in patients without shock. In addition, among patients with an unfavorable outcome, 75% of patients with septic shock died by day 5, whereas the same percentage of deaths was recorded by day 22 in patients without septic shock.

The significant influence on mortality rates of variables imperfectly balanced by the randomization process, led us to analyze the net effect of treatment on 30-day mortality after adjusting for the presence of covariates in a Cox regression model.[50] In addition to the presence of sepsis, septic shock and the requirement for hemodynamic support, the baseline MOF score and plasma AT activity, the time to treatment, age and center were included as covariates in the model (Table 5). At multivariate analysis, AT replacement had a net beneficial effect on 30-day survival (OR = 0.56, p<0.02). Of the covariates analyzed, the presence of septic shock (p=0.0002) and the baseline MOF score (p=0.02) were negatively associated with survival, while plasma AT activity levels (p=0.003) were positively and independently associated with survival, which also differed according to the center (p=0.006).

Because the two treatment arms were unbalanced for septic shock, the interaction of treatment with septic shock was tested in the model and resulted to be significantly associated with survival (p<0.0001). After stratification of patients for the presence or absence of septic shock, a net effect of AT treatment on mortality was observed only in the septic shock group (OR = 0.43, Table 5). In patients with septic shock, a significant effect of AT treatment on survival was also shown by Kaplan-Meier analysis (p = 0.04). Septic shock patients receiving treatment had a 34% (95% CI: 19%-49%) probability of being alive at day 30, with a corresponding probability of 13% (95% CI: 0%-26%) for septic shock patients receiving placebo and a resulting 30% reduction in 30-day mortality. The reduction in mortality produced by AT replacement was even more apparent when excluding early deaths from the analysis (p=0.016).

Table 5. Variables independently affecting survival of critically ill patients by Cox hazard regression analysis (ref. #11c).

Variables selected	*Odds Ratio*	*95% C.I.*
All patients		
Baseline AT % activity	0.97*	0.95-0.99
Center	1.61	1.15-2.24
Septic shock	3.97	1.77-6.25
Treatment	0.56	0.31-0.91
Patients with septic shock		
Baseline AT % activity	0.97*	0.95-0.99
Center	1.53	0.99-2.36
Treatment	0.43	0.23-0.83

The model includes as variables: treatment, MOF, SAPS, and AT levels at baseline, requirement for hemodynamic support, centers, time from admission to ICU until enrolment in the study, sepsis, septic shock, age.
**For unitary increase in % antithrombin activity.*

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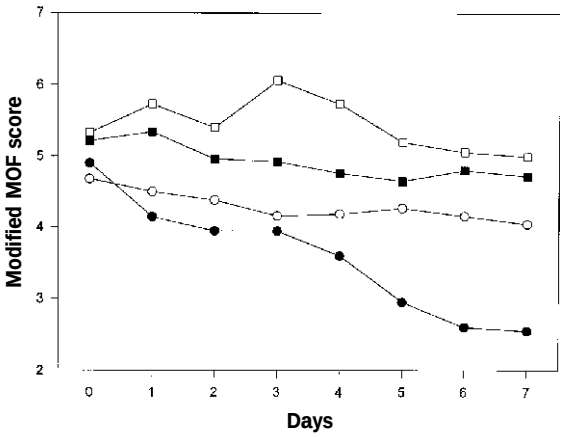

Figure 1. Adjusted changes in the modified MOF score after stratification of patients for the presence (squares) or absence (circles) of septic shock. Time (p=0.0001), presence of septic shock (p=0.002) and treatment allocation (AT replacement, closed symbols, versus placebo, open symbols, p=0.05) exerted independent significant effects on the modified MOF score.

In the entire population of patients, the MOF score, adjusted for its baseline value showed a significant change with time (p<0.001), but not with treatment (p=0.26). The differences observed in mortality rates and in the effect of treatment in patients with or without septic shock led to a revaluation of the changes in the MOF score during the first week of patients' observation after stratification for the presence or the absence of septic shock. This analysis showed an independent effect of AT replacement in improving the MOF score both in patients with and without septic shock (p=0.05, Figure 1).

In spite of the observation of a favorable effect of AT replacement therapy on 30-day survival, this effect was no longer significant when considering the overall survival not truncated at day 30. The presence of septic shock (OR = 3.42, p<0.001) and the baseline AT levels (OR = 0.98, p<0.05) were the only variables associated with overall survival, which was, however, also significantly different in the three centers participating in the study.

Conclusions and future perspectives

Patients admitted to intensive care units because of the requirement for hemodynamic and/or respiratory support are a highly heterogeneous group, with underlying diseases of different etiology. It would therefore be expected that addition of a single drug to the therapeutic strategy would hardly have a major effect on mortality. In spite of this limitation, a similar trend for a beneficial effect of AT treatment has been reported in small double-blind studies of critically ill patients hospitalized in intensive care units. Our findings in septic shock patients are similar to those of Fourrier *et al*.,[43] who aimed to produce and maintain very high levels of plasma AT activity for 5 days (175-200%). In their efficacy analysis treatment with AT resulted in normalization of laboratory parameters of DIFF in survivors within 10 days, and there was a 56% reduction in 30-day mortality in the active treatment arm. However, because of the low number of patients enrolled and of the relatively high overall survival rate (59%), this figure did not reach statistical significance. Lamy *et al*.[44] infused a total amount of 18,000 units of AT over 5 days obtaining a 52% reduction in 30-day mortality for septic shock. This figure, too, did not reach statistical significance because of the low number of patients enrolled and the high survival rate (67%). In our series of 56 septic shock patients, the survival rate was 25%, similar to the 19% survival rate reported in Italian intensive care units.[17]

The beneficial effect of AT replacement in septic shock patients is indirectly further supported by the observation of the independent negative prognostic value of low plasma antithrombin activity at enrolment into the study. The predictive value on outcome of baseline plasma AT activity has been shown in DIC of different etiologies and in chemotherapy-induced neutropenia in acute leukemia and lymphoma. In one study,[51] the development of septic shock in neutropenic patients was associated with early evidence of increased thrombin generation, and antithrombin levels lower than 70% at the onset of fever predicted a fatal outcome, with a sensitivity and specificity of 85%. Because the presence of AT levels <70% was a criterion for inclusion into our study, our findings point to the predictive value of AT in septic shock being independent of the severity of the disease (as also suggested by the non-influence of the baseline MOF score on survival) and they also indicate a causal relationship between the degree of activation of the coagulation mechanisms and the occurrence of death.

In spite of the beneficial effect of a 5-day course of AT replacement therapy, the overall mortality – not truncated at day 30 – was similar in patients receiving placebo or AT. The influence of AT replacement on laboratory markers of coagulation and fibrinolysis is currently being evaluated. It is possible that either the AT replacement protocol was insufficient to quench the activation of coagulation, or that approaches aimed at controlling inflammation may be required in addition to AT to obtain a prolonged effect on the survival of patients with septic shock. In animal models of septic shock, the administration of protein C had a clear-cut effect on survival.[28] Protein C concentrate administration has proven highly effective in reducing mortality of patients with meningococcus-induced *purpura fulminans*,[52] a syndrome characterized by very low levels of protein C activity and antigen.[53] Because protein C has both anticoagulant and anti-inflammatory properties,[54] future studies should evaluate the effect of the combination of AT and protein C administration on the survival of patients with septic shock.

Contributions and Acknowledgments

DG and ADA wrote the manuscript; FB, GP, AR and LR participated in the design of the study which is the major issue of the present review and they are listed in alphabetical order. All the authors read and approved the final version of this manuscript.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received November 6, 1998; accepted January 26, 1999.

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Richter's syndrome in a patient with B prolymphocytic leukemia

MARIE-JOSÉ GRANGE,* VALÉRIE ANDRIEU,* KADOUDJA CHEMLAL°
*Departments of *Hematology, °Internal Medicine, Hôpital Bichat, Paris, France*

A 63 year-old previously healthy man presented with marked lymphocytosis. Physical examination showed splenomegaly but no adenopathy. The blood count was 38.5×10^9 leukocytes with 88% lymphoid cells, 170 g/L hemoglobin and 246×10^9/L platelets. The lymphoid cells were large with a single thick-rimmed nucleolus in a round regular nucleus. The chromatin was moderately dense and the cytoplasm was pale blue (Figure 1). Cytological diagnosis was prolymphocytic leukemia (PLL). Immunophenotyping of lymphoid cells by flow cytometry showed a B proliferation $CD19^+$, $CD5^+$, $CD23^+$, CD24, Fm $C7^-$, $CD79^+$ with strong expression of surface IgM κ. Cytogenetic study of blood cells was normal without 14:18 translocation and cyclin D1 was not expressed.

The patient was asymptomatic for several months before developing, in a few days, enlarged, tumoral, cervical lymph nodes. Cytological examination of these lymphadenopathies showed voluminous cells: their nuclei had prominent nucleoli and very thin chromatin. The cytoplasm was deep blue (Figure 2). A diagnosis of immunoblastic lymphoma was confirmed by histological examination.

This patient appears to have Richter's syndrome, that is an aggressive lymphoma which occurred several months after the diagnosis of lymphocytic leukemia. We re-assessed the diagnosis of B-PLL with regard to the cytologic aspect of the lymphoid cells and the strong expression of sIg despite the negativity of FmC7. Richter's syndrome occurs in about 5% of patients with chronic lymphoid leukemia, but to our knowledge, has not been reported in patients with PLL.

Correspondence: Kadoudja Chemlal, M.D., Service de Médecine Interne, Hôpital Bichat, 46, rue Henri Huchard, 75877 Paris Cedex 18, France. Phone: international +33-1-40257001 – Fax: international +33-1-40258845.

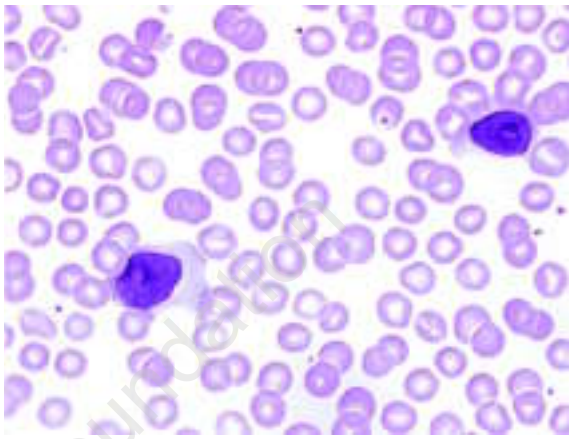

Figure 1. Peripheral blood prolymphocytes with prominent nucleoli (MGG ×1000).

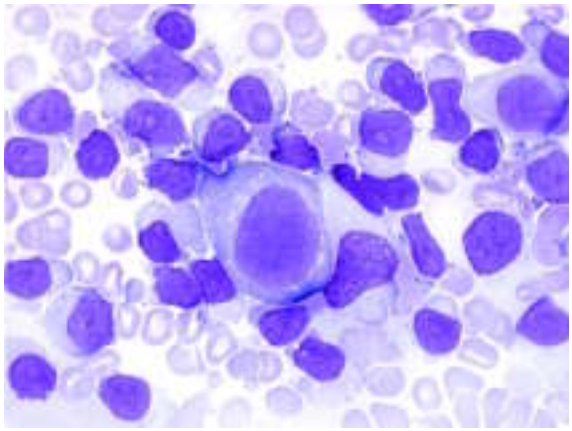

Figure 2. Cytologic examination of a cervical node showing immunoblasts (MGG ×1000).

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Haematologica 1999; 84:462-463 the irreplaceable image

Acenocoumarol-induced leukocytoclastic vasculitis

FRANCISCO JOSÉ JIMÉNEZ-GONZALO,* MANUEL MEDINA-PÉREZ,° JORGE MARÍN-MARTÍN#
**Servicio de Hematología y Hemoterapia;°Servicio de Anatomía Patológica; #Servicio de Medicina Interna, Hospital de la Merced, Osuna, Seville, Spain*

We report a case of a acenocoumarol (Sintrom®)-induced vasculitis in a 54-year-old white female receiving oral anticoagulant therapy for atrial fibrillation, secondary to mitral stenosis. She was given digoxin, furosemide and acenocoumarol. In the third week of acenocoumarol treatment the patient was feverish (38ºC) and both legs became tender and swollen. The skin was initially erythematous and later showed purpuric lesions and hemorrhagic bullae (Figure 1). Histologic examination of the involved skin demonstrated vascular changes of small vessels with fibrinoid necrosis of the walls. Polymorphonuclear leukocytes were found within and around the vessel wall with many scattered nuclear fragments (leukocytoclastic vasculitis) (Figure 2). The INR, being 2.35, was within the normal therapeutic range. Antinuclear antibody and anti-neutrophil cytoplasmic antibody tests were negative. Two months after withdrawal of acenocoumarol, the levels of protein C and S (total and free) were determined, being 88%, 78% and 70%, respectively. The coumarin was stopped and prednisone was started at a dose of 1 mg/kg/day IV. The skin lesions and fever resolved. The patient refused treatment with another coumarin drug and she was discharged on digoxin, furosemide and aspirin (100 mg/day).

Anticoagulants of the coumarin family are frequently administered for the treatment and prophylaxis of arterial and venous thromboembolic disease. The most common side effect of these drugs is bleeding due to excessive lowering of the procoagulant factors, but uncommon complications such as skin reactions have been described.[1] Among the cutaneous side effects of oral anticoagulant therapy, the most frequent are the *purple toes syndrome* and warfarin-induced skin necrosis,[2] although hypersensitivity reactions such as vasculitis are possible and have been previously described in several reports.[3-6] In severe cases of vasculitis, the cutaneous manifestations may mimic skin necrosis: erythematous lesions that expand and evolve into red-purple zones with hemorrhagic bullae. In this situation, it is important to make a differential diag-

Correspondence: F.J. Jiménez Gonzalo, M.D., Servicio de Hematología y Hemoterapia, Hospital de la Merced, Avenida de la Constitución 2, 41640 Osuna, Seville, Spain. Phone: international +34-95-5820920 – Fax: international +34-95-5820544 – E-mail: francisjg@arrakis.es

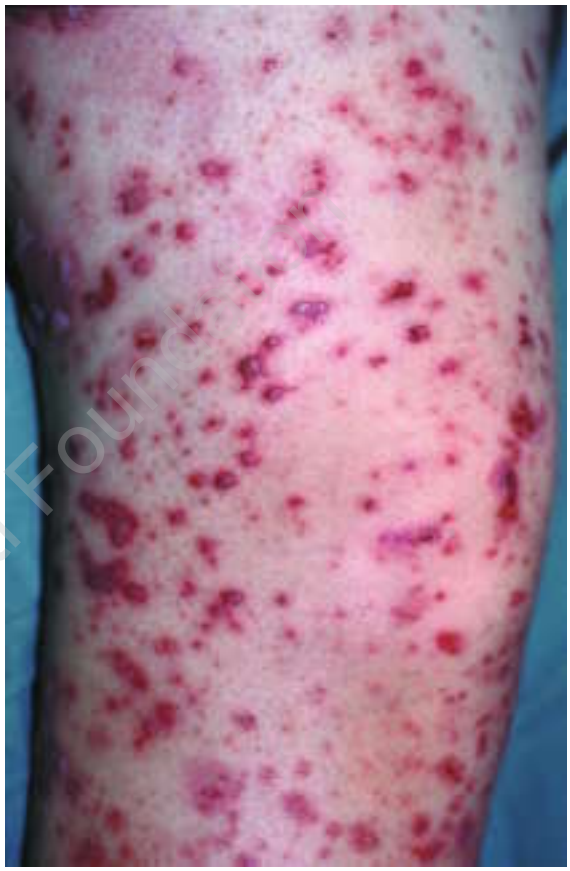

Figure 1. Hemorrhagic lesions with surrounding normal tissue.

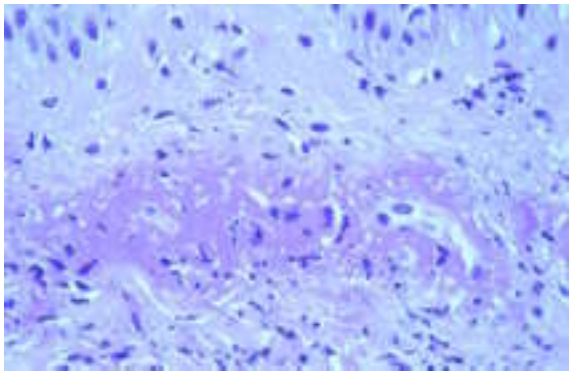

Figure 2. Skin biopsy. Fibrinoid necrosis of the vessel wall with infiltration by neutrophils and nuclear fragments (H&E 400×).

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nosis from warfarin-induced skin necrosis, because the therapy of the two entities is different.

Warfarin-induced skin necrosis typically presents between the third and eighth day of therapy, although it can appear later.[7] It is more common in females, and frequently affects areas where there is abundant subcutaneous fatty tissue: breast, thighs and buttocks. In males the penis is affected, while the breast is spared. Histopathologically, there is microvascular thrombosis in the areas of skin involved. Declining protein C and S levels are thought to be important in the pathogenesis because of a rapid fall in the levels of these anticoagulant proteins before the anticoagulant effect is obtained, although other factors may be involved.[8-10] By contrast, vasculitis appears later, is accompanied by an inflammatory infiltrate, its localization is different and the treatment is based on discontinuation of the drug plus administration of steroids.

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A retrospective evaluation of infant patients with acute lymphoblastic leukemia treated at a single institution

Sir,

We report a retrospective evaluation of 10 infants with acute lymphoblastic leukemia (ALL), diagnosed and treated at a single institution, who achieved a 10-year acturial event free survival of 50%.

Infant acute leukemia (AL) is a distinct leukemic subset with an extremely poor prognosis, having a 25% event free survival (EFS) at 4 years.[1-6]

Rearrangements of the ALL1/MLL gene at 11q23 cytogenetic band[7,8] have been demonstrated in 70% of infants. Recently, this molecular alteration has been associated with an adverse clinical outcome.[7,9]

Biological and clinical features, treatments, and outcome of 10 ALL infants, observed and treated at the Children's Hospital "Bambino Gesù" of Vatican City State between August 1986 and September 1996, are summarized in Table 1.

The genomic ALL1/MLL configuration was investigated in 4 patients for whom stored material was available. Three of them showed an ALL1 rearrangement, while the fourth had a t(4:11)(q21;q23) translocation.

Patients were treated according to the then current treatment protocols of the Italiana Association of Pediatric Oncology and Hematology (AIEOP).[10]

Two of 4 patients treated with LA84 INF, which includes high dose methotrexate (6 g/m^2), are in first complete remission (CR) at 84 and 108 months from diagnosis; the remaining two cases are in second CR after extra-hematologic relapses (testis, CNS) which occurred at 110 and 70 months from diagnosis.

All 5 patients treated with the AIEOP 9102, 9103, 9502 protocols (BFM like) achieved CR. Three out of 5 patients are still in first CR at 17, 17 and 26 months; the fourth patient had a CNS relapse after 8 months of CR, and is now in second CR 24 months after an allogeneic bone marrow transplantation; the fifth patient had a hematologic relapse and died of his disease 11 months after diagnosis. Finally, the case with t(4;11) failed to respond to an induction treatment which included adriamycin, cytarabine and prednisone. As shown in Figure 1, the 9-year EFS and OS were 50% and 80%, respectively.

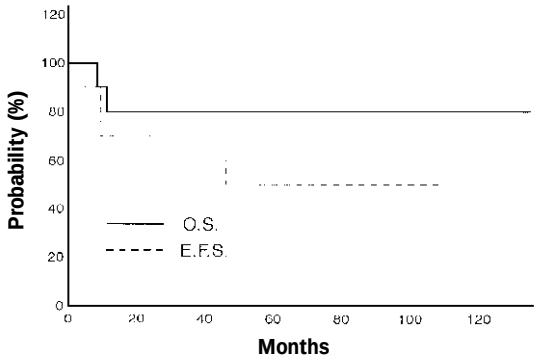

Figure 1.

Casual selection of infants with less aggressive disease is probably the most plausible explanation of our observed favorable results. In fact, we detected a low incidence of pre-B and hybrid immunophenotypes. Six patients had a normal karyotype, while a 11q23 cytogenetic alteration and/or ALL1/MLL rearrangements were demonstrated in only four cases. This hypothesis is further supported by the observation that our ALL1/11q23 rearranged cases did less well than the other patients. In contrast, the fact that patient #3, who presented at diagnosis with 660×10^9/L white blood cells but had an ALL1 germline configuration, is actually cured of his disease, indirectly confirms that ALL-1/MLL rearrangement is the most important adverse prognostic factor in infant ALL.

Table 1. Clinical and biological features as well as type of treatment and therapeutic response in 10 infants with ALL.

Pat.	Age (months)	Sex	WBC (10^9/L)	Immuno-phenotype	Cytogenetic analysis	DNA analysis	Therapy	1st CR	Relapse	2nd CR	Follow-up (months)
1	8	F	28	T-ALL	46 XY	ND	LA 84, INF	yes	yes (CNS)	yes	135 +
2	4	M	85	ND	46XY	ND	LA 84, INF	yes	yes (testis)	yes	112+
3	10	M	660	T-ALL	46XY	ND	LA 84, INF	yes	-	-	108+
4	7	M	15	Common	+8	ND	LA 84, INF	yes	-	-	84+
5	2 days	F	68	Common	t(4;11)	ND	ARA-c, ADM, PDN	no	-	-	8
6	4	F	21	Common	46XX	ND	AIEOP 9102	yes	yes (CNS)	yes	36+
7	3	F	17	Hybrid	t(4;11)	ALL1 rearr.	AIEOP 9103	yes	yes (BM)	-	11
8	1 day	F	90	Common	del (10 p)	ALL1 rearr.	AIEOP 9502	yes	-	-	26+
9	10	M	150	Pre-B	46 XY	ALL1rearr.	AIEOP 9502	yes	-	-	17+
10	10	M	23	Pre-B	46 XY	ALL1 g l	AIEOP 9502	yes	-	-	17+

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As to toxicity, only one patient, who received a total anthracycline dose of 240 mg/m^2, developed a severe dilated myocardiopathy.

In conclusion our results confirm that some infants with ALL can be cured by conventional chemotherapy. Thus, we believe that careful stratification for prognosis is needed to treat these patients with adequate risk-adapted therapeutic strategies.

Matteo Luciani, Giuseppe Cimino,# Adriano Angioni,° Lidia Angiolina Russo,* Teresa Sprovieri,# Giulio De Rossi**

**Divisione di Ematologia, °Laboratorio di Citogenetica e di Tipizzazione cellulare, Ospedale Pediatrico "Bambino Gesù", Città del Vaticano; #Dipartimento di Biotecnologie Cellulari ed Ematologia, Divisione di Ematologia, Università "La Sapienza" di Roma, Italy*

Key words
Childhood, acute lymphoblastic leukemia

Correspondence
Matteo Luciani, MD, Divisione di Ematologia, Ospedale Pediatrico "Bambino Gesù", Città del Vaticano, piazza Sant'Onofrio 4, 00165 Rome, Italy.

Phone: international +39-06-68592129 – Fax: international +39-06-68592392.

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Three cases of Kikuchi-Fujimoto disease

Sir,

Kikuchi-Fujimoto disease (KFD) is a benign form of necrotizing lymphadenitis of unknown cause, usually affecting young women[1,2] and characterized by lymphadenopathy often associated with fever and leukopenia. Cervical nodes are usually involved, while generalized lymphadenopathy, hepato-splenomegaly and extranodal involvement are uncommon. Laboratory tests show only a raised ESR.[3] The diagnosis is based on distinctive lymph node histologic features:[4,5] focal necrosis in cortical and paracortical areas; karyorrhectic nuclear debris mixed with a polymorphous cell population including immunoblasts and histiocytoid cells. Polymorphonuclear leukocytes and B cells are characteristically absent. In early phases T-suppressor lymphocytes are predominant. Lesions in different stages of development may coexist; focally, the histologic pattern might be mistaken for lymphoma or other diseases.[4-6] The disease resolves spontaneously within 2-3 months; relapse is not common. For unknown reasons, this disease is more frequent in Japan, where it was first described.

In a five-year period, we have observed three patients with KFD.

Case #1. A 36-year-old woman presented in January 1993 with fever and cervical lymphadenopathy, unresponsive to antibiotics and low dose prednisone. The ESR was 70 mm, all other laboratory tests were negative. Bone marrow examination was normal. Lymph node histology was typical of KFD (Figure 1). The patient recovered in three weeks with no other treatment.

Case #2. A diabetic 53-year-old man was observed in December 1996 with a four-month history of fatigue, fever, and moderate cervical and axillary lymph node enlargement. His ESR was 88 mm, all other tests were normal. Bone marrow examination showed a normocellular marrow. Lymph node biopsy demonstrated KFD. He received antibiotic therapy after biopsy, and the disease resolved over four weeks.

Case #3. A 31-year-old woman was admitted in March 1997 because of fatigue, fever, night sweats, cough and cervical lymph node enlargement. Laboratory tests were all normal, including ESR. Lymph node biopsy showed necrotizing lymphadenitis of the KFD type. She recovered in six weeks with no treatment (Table 1).

The etiopathogenesis of KFD is still obscure. Some clinical and histologic features suggest a possible infectious etiology, particularly viral.[3,7] It has also been supposed that KFD may have different etiologies, all provoking an abnormal cell-mediated immune response. This hypothesis is supported by the finding of cytoplasmic tubuloreticular structures; similar structures are observed in other diseases, particularly systemic lupus erythematosus (SLE).[3] KFD and SLE may be associated and KFD may precede the onset of

Table 1. Clinical features.

Pts	Sex/Age	Involved Nodes	Symptoms	Abnormal laboratory test	Therapy	Time from diagnosis to recovery
1	F/36	Left, cervical	Fever	ESR	Antibiotic+steroids	Six weeks
2	M/53	Cervical, axillary	Fever, fatigue	ESR	Antibiotic	Four weeks
3	F/31	Cervical	Fever, fatigue, sweats, cough	–	–	Six weeks

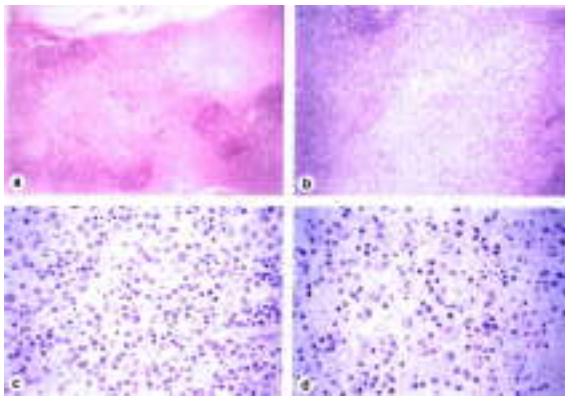

Figure 1 (left). Typical lymph node histology of Kikuchi-Fujimoto disease (case #1).
a) lymphoid tissue with multiple nodular necrotic foci; b) a necrotic area adjacent to a normal follicle; c) a necrotizing lesion with karryorrhectic nuclear debris; phagocytizing histiocytes and a few lymphocytes; neutrophils are absent; d) a necrotic area containing lymphocytes, histiocytes and nuclear debris.

SLE.[3,8-9] Therefore, prolonged follow-up of patients with KFD to reveal possible connections with SLE is suggested. The prevalence of the disease may be underestimated, since the spontaneous regression of the symptoms may hinder the diagnosis in some cases. All our patients were resident in rural areas surrounding the city, 20-30 km away from each other. We have found only two other cases of KFD from Italy reported in the literature; one was a patient with AIDS.[4,10] Thus, no estimate of the incidence of KFD in Italian populations can be attempted. We do not know whether the identification of three cases over a few years in a relatively restricted area is a casual event, possibly related to a greater attention to this disease, or whether it indicates the existence of local factors responsible for an endemic spread. Our patients recovered in about six weeks; neither relapse nor onset of other diseases has been observed with follow-ups of sixty-three, fifteen and thirteen months, respectively.

Lidia F.E. Santoro, Amalia De Renzo, Renato Silvestri, Gennaro De Rosa, Guido Pettinato,* Bruno Rotoli*

*Division of Hematology, "Federico II" University Medical School; *Department of Pathology, Division of Internal Medicine Fatebenefratelli Hospital, Naples, Italy*

Key words

Adenopathy, necrotizing lymphadenitis, Kikuchi-Fujimoto disease

Correspondence

Bruno Rotoli, M.D., viale Augusto 148, 80125 Naples, Italy. Phone: international +39-081-7462068 – Fax: international +39-081-7462165.

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Bernardo Ferrari Foundation

α-interferon as induction and maintenance therapy in hairy-cell leukemia: a long-term follow-up analysis

Sir,

The treatment of hairy cell leukemia (HCL) has improved greatly over the last two decades thanks to the introduction of first IFN[1,2] and later new purine analogs.[3,4] In particular the latter have revolutionized prognosis of HCL by increasing the number of long term complete responses.[5] The real impact on overall survival, however, is not yet clear. In this study the long term outcome of a cohort of patients treated with IFN is reported. Sixty-four patients observed between 1980 and 1996 with a histologic diagnosis of HCL, including 11 patients who had undergone splenectomy, received IFN 3MU ×3/week for 12-18 months as first line treatment. The overall response rate was 91%. Sixty-five percent achieved PR and 26% went beyond PR (13% CR and 13% GPR) (Table 1). Forty-one patients (71%) were administered IFN 3 MU/week as maintenance therapy after first line therapy (Table 1). After IFN induction therapy 20 patients relapsed and were retreated (8 under maintenance). Twenty-six patients received a second course of treatment (6 non responders and 20 relapsed after first line therapy). IFN, 2CdA, and DCF were similarly effective, with 2CdA and DCF producing higher CR and GPR rates than IFN. IFN as second line therapy (employed in patients who had previously responded to IFN) produced an overall response rate (90%) identical to that which had been observed in first line therapy. In October 1998 the median follow-up for surviving patients was 97 months. Figure 1 reports the ten year projected survival of patients achieving objective response to first line therapy (CR and GPR 100%; PR 95%) and that of non responders (NR) and clearly shows that the type of response does not affect survival.

Figure 2 shows that patients receiving IFN maintenance after IFN first line therapy had a statistically higher PFS than those not receiving maintenance therapy ($p < 0.01$).

During the last few years 2-CdA has emerged as the treatment of choice for HCL patients. However the possible effects of the profound and protracted immunosuppression caused by the drug are not completely known, thus caution is required.[6]

The results of our experience confirm that IFN is still an excellent treatment for HCL patients, producing 91% of objective responses, while having a low toxicity. In particular, IFN is not associated with an increased incidence of autoimmune diseases . As already pointed out,[7] IFN induced responses were mostly partial. The type of response does not, however, affect survival since patients achieving CR/GPR and PR had a ten year projected survival probability of 100% and 95%, respectively. The series by Rai[8] and

Table 1. Patients' features and outcome.

No.	64*	
M/F	66/16	
Mean age (range)	52	(23-73)
Spleen size - cm under costal rib (range)	8	(0-24)
% pts with splenomegaly	55	
Hb g/dL	11	(4-14)
Ht	33	(10-46)
% pts with Hb < 10 g/dL	40	
WBC $\times 10^9$/L (range)	5	(1-17)
% pts. with WBC < 3×10^9/L	51	
Plt $\times 10^9$/L (range)	93	(20- 390)
% pts with Plt < 100×10^9/L	68	
PMN % (range)	27	(6-55)
L % (range)	58	(9-83)
M %(range)	6	(2-15)
% peripheral blood HCs (range)	32	(0-87)
% pts with HCs in peripheral blood	50	
% Bone marrow cellularity (range)	56	(5-92)
% Bone marrow HC infiltration (range)	50	(5-95)
HCI (range)	0.31	(0.01-1)
NR	6(9%)	
Overall responses	58 (91%)	
PR	42 (65%)	
GPR	8 (13%)	
CR	8 (13%)	
PR-GPR-CR progression free period°	41-59-71	
Mean survival of PR-GPR-CR (months)	76-91-100	
Mean survival of SD-PD	57	

**preceded by splenectomy in 11; °41/58 (71%) received IFN maintenance therapy: response duration in maintained pts = 65 months (7-144); response duration in non maintained pts: = 42 months (9-72). HCI = hairy cell index; GPR = good partial response.*

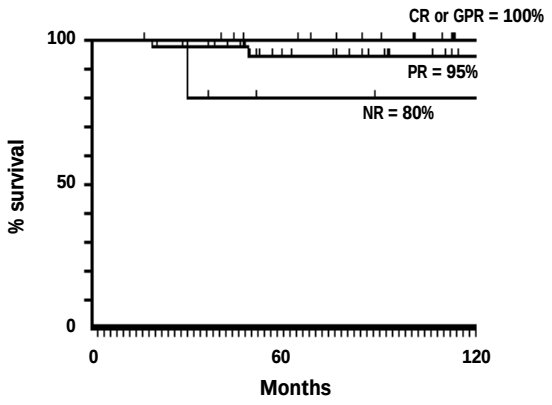

Figure 1. Survival according to type of response to first line IFN therapy.

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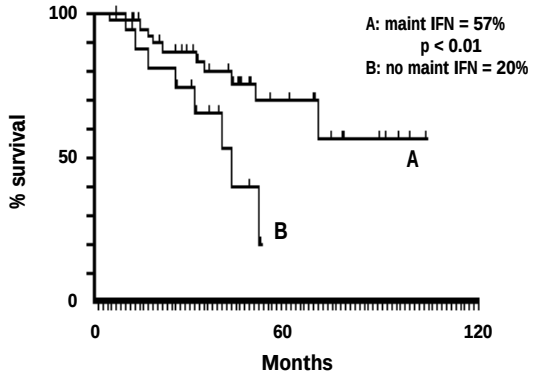

Figure 2. Progression free survival after first line IFN therapy, according to IFN maintenance therapy.

Zaja[9] also yielded similar results. A statistically significant lengthening of PFS was the result of IFN maintenance therapy, as already reported.[10]

Our experience, although limited, confirms the efficacy of purine analogs as second line therapy.

In agreement with Rai *et al*.[8] the very high projected survival probability of our series can be explained mainly by a two step therapeutic strategy made up of IFN first line therapy and purine analog salvage treatment for IFN resistant patients. Considering on the one hand our results and the reported good outcome of patients receiving IFN first,[7,8] and, on the other hand, the high CR rate and the prolonged survival obtained with front line purine analog therapy, the problem of defining a recommended first line therapy for HCL remains unsolved.

In conclusion, although the therapeutic emphasis in HCL has recently shifted to 2-CdA and DCF, IFN remains a therapeutic choice for this disease. We confirm that IFN is effective and tolerable and prolonged, reduced dosage administration may produce a long progression free period. We also confirm that achieving CR has no primary relevance in disease control and that good use of therapeutic resources may assure HCL patients a survival which is comparable to that of the normal, healthy population.

Marino Clavio, Bahman Masoudi, Mauro Spriano,* Salvatore Casciaro, Marco Gobbi, Eugenio E. Damasio**

**I Division of Hematology, Chair of Hematology DIMI, Azienda Ospedale S. Martino and Cliniche Universitarie Convenzionate, Genoa, Italy*

Key words

Hairy cell leukemia, α-interferon

Correspondence

E.E. Damasio, I Division of Hematology, Azienda Ospedale S. Martino e Cliniche Universitarie Convenzionate, largo R. Benzi 10, 16132 Genoa, Italy. Phone +39-010-5552557. Fax +39-010-5556609 – E-mail: edamasio@smartino.ge.it

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Screening for hemoglobinopathies in neonates in Argentina

Sir,

So far there is very little published information about the distribution of hemoglobinopathies in Argentina.[1] Historically the Argentine population is predominantly composed of individuals of Spanish, Aboriginal and mixed ancestry. There was an important wave of Italian immigration between 1876-1925, when 2,145,000 Italians arrived in our country, and another between 1947-1951 when a further 400,000 arrived. These immigrants settled especially in the provinces of Buenos Aires, Santa Fe, Cordoba, Mendoza and Entre Rios.[2] The population under study comes from the city of Rosario, in the south of Santa Fe.

One thousand unselected, umbilical cord blood samples collected with EDTA from consecutive neonates (Hospital Provincial del Centenario), were submitted daily to: identification of unstable hemo-

Table 1. Hereditary hemoglobin disorders in 1000 neonates.

Diagnosis	*Abnormal Hb (%)*	*N*	*Percentage (%)*
FA	–	993	99.3
FAS	8.0	5	0.5
Unstable-FA	2.9	1	0.1
FA-Bart	4.4	1	0.1
Total	–	1,000	100.00

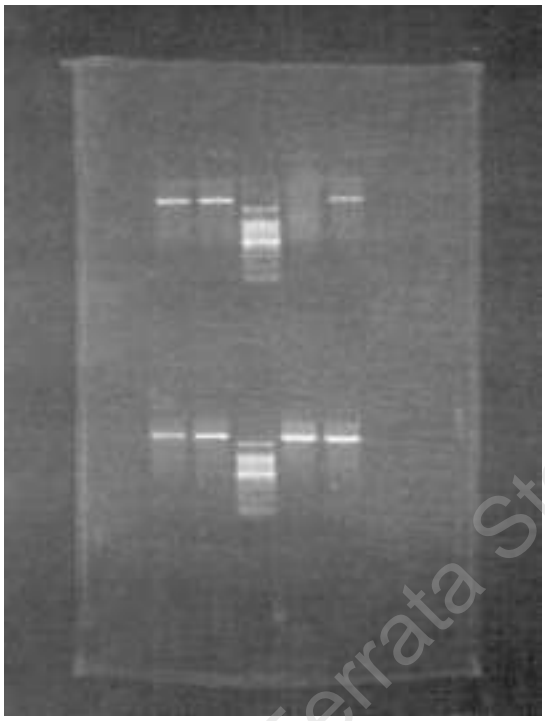

Figure 1 Detection of 3.7 Kb α-thalassemia-2 deletion by PCR. Lanes 1, 2: control α-thalassemia-2 (-3.7 Kb) heterozygote (αα/-α); lanes 3, 8: marker 100 bp ladder; lanes 4, 5: FA-Bart cord blood; lane 6, 7: father; lanes 9, 10: mother. Lanes 1, 4, 6, 9 using normal primers; lanes 2, 5, 7, 10 using specific primers for α-thalassemia-2 (-3.7 Kb).

globin by thermostability tests; detection of inclusion bodies. The hemolysate was analyzed by electrophoresis in alkaline and acid media. Whenever HbS was detected, induction of drepanocytosis was performed. The identification of chains by HPLC[3] was assessed in the unstable Hb. In the sample with Hb Bart, the presence of α^{+}Th was assessed by PCR.[4]

Data are presented in Table 1. The FAS phenotypes yielded a positive drepanocyte test, they were classified as heterozygous for Hb S, confirmed by family study.

An $\alpha^{3.7}$ homozygous deletion was identified, which was also detected in the proband's parents in a heterozygous state (Figure 1). The only positive unstable-FA was also detected in the neonates's mother (11%). It was electrophoretically silent, but with HPLC we determined that the anomalous chain was the β globin.

Cord blood screening is a useful tool for identifying sickle cell disease and α-thalassaemia. The population under study was Caucasian, and constituted mainly of individuals of Spanish origin, including a high percentage of mixed ancestry (Spanish-Aboriginal), and a minor proportion of individuals of Italian origin, and others. The early detection of HbS is of the utmost importance to reduce morbidity and mortality during the first years of life due to sickle-cell disease complications,[5] such as frequent infections. The risk of these complications increased even in heterozygous cases. Five HbS carriers were detected in our study: one of Italian ancestry and four of mixed ancestry. The observed heterozygote rate (1:200) is consistent with the gene frequency in the Mediterranean region.[6] The introduction of the gene S into our population is probably due to Spanish and Italian immigrants, since HbS in New World Amerindians has never been reported.[7]

In the newborn child (of Spanish ancestry) with Hb Bart the presence of an $\alpha^{3.7}$ deletion was investigated for, since this is frequent in Spain and Italy. It is probable that cases with the -α/αα genotype failed to manifest Hb Bart,[8] since the only positive result was homozygous (-α/-α).

Our results allow us to conclude that although screening studies in neonates are routinely carried out in several countries,[9,10] the low frequency of hemoglobinopathies detected in the present study does not justify these routine studies in our population. These results do, however, shed light on the prevalence of hemoglobinopathies in our region since up to the present time there are no published data on neonates in Argentina.

Nélida I. Noguera, Irma M. Bragós, Lida Morisoli, Angela C. Milani

Area Hematología. Departamento Bioquímica Clínica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (UNR), Rosario, Argentina

Key words

Hemoglobinopathies, neonates, screening

Acknowledgments

We are very grateful to the professional staff of the Cátedra de Hematología, Departamento de Bioquímica Clínica, Fac. de Cs. Bioquímicas y Farmacéuticas, UNR, to Miss Analía Servidio for her technical assistance, and to the staff of the Obstetrics Department, Hospital Provincial Centenario for their cooperation in the sample collection.

Correspondence

Nelida I. Noguera, M.D., Area Hematología - Departamento Bioquímica Clínica, Facultad de Ciencias Bioquímicas

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y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531-2000 Rosario, Argentina. Phone: international +54-41-372704 – Fax: international + 54-41-804598 – E-mail: nnoguera@fbioyf.unr.edu.ar

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Patients with venous thromboembolism have a lower APC response than controls. Should this be regarded as a continuous risk factor for venous thrombosis?

Sir,

Activated protein C (APC) resistance is characterized by a poor anticoagulant response to APC.[1,2] In most cases it is caused by the factor V Leiden mutation (FVLm) (1,691G→A).[3] Inherited APC-resistance has been found in 15-40% of thrombotic patients.[4] We report the APC-response of a group of thrombotic patients, the prevalence of APC-resistance and its thrombotic risk.

We studied 186 thrombotic patients (104 female, 82 male), referred to our Unit from January 1994 to March 1997. The clinical characteristics of the thrombotic individuals are shown in Table 1. The control group comprised 103 healthy blood donors (57 male, 46 female). Blood was collected 3-6 months after the most recent thrombotic event without influence of oral anticoagulants. APC-resistance was measured using a kit from Chromogenix (Möndal, Sweden). Antithrombin, protein C, S and lupus anticoagulant (LA) were also analyzed. Detection of FVLm was performed as described elsewhere.[3] Sex differences and influence of age were assessed by the chi-square test and correlation analysis. APC-ratios were compared by ANOVA, including age and sex as covariants. A logistic regression model was employed to estimate the odds ratio (OR), and to evaluate the risk of thrombosis associated with APC-resistance. The normal range was defined as the 2.5 and 97.5 percentiles (2.08-3.95). Patients had lower APC-ratios than controls (Figure 1) (difference after exclusion of APC-resistant individuals: 0.15, 95% CI: 0.03-0.27, p<0.05). Females had lower APC-ratios (difference after exclusion of APC-resistant individuals 0.20, 95% CI: 0.092-0.30, p<0.0001). No age influence was observed.

Patients with APC-resistance showed more than a five-fold increase in risk of thrombosis (OR 5.4; 95% CI: 1.8-16.4, adjusted for age and sex). A tendency towards an inverse relationship between the risk of thrombosis and the degree of APC-response was found [APC-ratio <2.08, OR 6.25 (95% CI: 2.01-19.42); APC-ratio 2.08-2.50, OR 1.82 (95% CI: 0.92-3.59); APC-ratio >2.5 was the reference interval].

Table 1. Clinical characteristics of the 186 thrombotic patients.

	n (%)
Sex	
male	82 (44)
female	104 (56)
Family history of thrombosis	77 (41)
Age at first thrombosis (mean±SD)	42.8±15.6
Spontaneous	67 (36)
Secondary*	119 (64)
orthopedic surgery	18 (9.7)
abdominal surgery	20 (10.8)
gynecological surgery	8 (4.3)
immobilization	8 (20.4)
pregnancy°	17 (16.3)
oral contraceptives°	14 (13.5)
varicose veins	12 (6.5)
neoplasms	8 (4.3)
others	15 (8.1)
Site of thrombosis	
deep vein thrombosis	109 (58.6)
pulmonary embolism#	53 (28.5)
superficial thrombophlebitis	14 (7.5)
upper arm thrombosis	5 (2.7)
mesenteric thrombosis	4 (2.2)
intracranial vein thrombosis	1 (0.5)

**Some patients had more than one risk factor (percentage of all cases); °only women were considered; #deep vein thrombosis was diagnosed in 36 patients with pulmonary embolism.*

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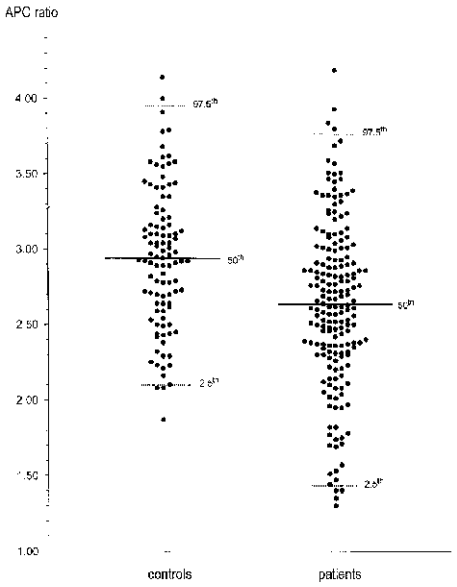

Figure 1. Anticoagulant response to APC in controls and patients with thrombosis. The response to APC was determined by the APC-resistance test, and the results were plotted as APC-ratios. Each person is represented by a full circle. The 2.5, 50 and 97.5 percentiles are indicated.

Activated partial thromboplastin time (APTT) was lower in patients and was inversely related to age, but we found that APTT-ratios and APC-ratios were independent.

Four controls and 29 patients had APC-resistance [prevalence 3.9% (95% CI: 1.1-9.6) and 15.6% (95% CI: 10.4-20.8) respectively]. When 6 patients with LA were excluded, the prevalence decreased to 12.8% (95% CI: 7.9-17.7). Other prothrombotic abnormalities were identified in 17 patients (2 antithrombin, 2 protein C and 13 protein S deficiencies). One patient with PS deficiency had APC-resistance and carried the FVLm. Two heterozygotes were identified in the control group (2/103, prevalence of 1.9%, 95% CI: 2.4-6.8). Seventeen out of 22 APC-resistant patients without LA were heterozygotes (77.3%; 95% CI: 54.6-92.2).

Although thrombosis is common, inherited deficiencies of anticoagulant proteins are unusual.[5,6] APC-resistance is probably the most frequent abnormality in patients with thrombophilia.[1] Despite the fact that the prevalence of APC-resistance in our region is lower than in other European areas,[2,4,7] it was the most common defect until we found that the prevalence of the prothrombin 20210A allele was 17.2%.[8] Unfortunately, we were not able to detect this variant retrospectively in our patients. Our patients had lower APC-ratios than controls even after the exclusion of APC-resistant subjects. An acute-phase response effect has been suggested[2,9,10] but this was not the case with our patients. Another possibility is the existence of genetic or acquired abnormalities that could contribute to APC-resistance. In agreement with other authors,[4] we found a tendency towards a relationship between thrombotic risk and APC-ratios. This suggests that APC-resistance should be regarded as a continuous variable that increases thrombotic risk. Further studies are required to ascertain whether a reduced response to APC is associated with an increased risk of thromboembolism, regardless of the presence of FVLm.

Isabel Tirado, José Mateo,* Artur Oliver,° Montserrat Borrell,* Juan Carlos Souto,* Jordi Fontcuberta**

**Department of Hematology, Hospital Sant Pau, Barcelona; °Department of Hematology, Fundació Puigvert, Barcelona, Spain*

Key words

Venous thromboembolism, APC resistance

Acknowledgments

We are indebted to Cristina Vallvé, Teresa Urrutia, and Joaquim Murillo for their technical work and to George von Knorring for his assistance in the preparation of the manuscript. This work was partially supported by grants FIS-96/2189E and FIS-97/2032, from the Fondo de Investigaciones Sanitarias, Spain.

Correspondence

Jordi Fontcuberta, M.D., Department of Hematology, Hospital de la Santa Creu i Sant Pau, C/ Sant Antoni Mª Claret, 167, 08025-Barcelona, Spain. Phone: international +34-93-2919193 – Fax: international +34-93-2919192 – e-mail: jmateo@santpau.es

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Familial neurofibromatosis type I and adult acute lymphocytic leukemia

Sir,

Individuals with neurofibromatosis type I (NF1; von Recklinghausen's disease) are predisposed to certain cancers. Children are at increased risk of developing benign and malignant solid tumors (mostly neural tumors) as well as hematologic malignancies including juvenile myelomonocytic leukemia (a rare hematologic malignancy that affects patients less than 4 years of age and that is sometimes associated with monosomy 7), the monosomy 7 myelodysplastic syndrome and acute myelogenous leukemia.[1] The risk of young children with NFI developing a malignant myeloid disorder is 200 to 500 greater than the normal risk[2] and several lines of evidence support the notion that the loss or mutation of the NF1 gene (a tumor suppressor gene) deregulates the Ras pathway which is responsible for the leukemogenesis in these children.[3] The relative risks for non-Hodgkin's lymphoma and acute lymphocytic leukemia (ALL) were increased in one series[2] but the risk of developing ALL was not increased in two other reports.[4,5]

In contrast to the situation in childhood, the association between NF1 and malignant blood disorders has not been demonstrated in adulthood.[3,6,7] A Medline® search of reports from the last 10 years uncovered only 2 adult patients with NF1 who developed an acute myelogenous leukemia[6,8] and none an ALL

It is, therefore, of considerable interest that we have seen 2 cases of adult-ALL, diagnosed over the last 12 months, in patients with familial NF1. This represents a 1.1% incidence in 176 ALL in patients over the age of 14 years seen in our Service during the last 25 years.

The diagnosis of NF1 is mainly clinical (*café-au-lait* spots, freckling, cutaneous neurofibromas, lentigo, Lisch's nodules). Our two patients had these signs and several relatives with the disease (Table 1) . Case #1 was a 19 year-old male with a familial history of NF1 (mother and the only sister affected) and case #2 a 31 year-old woman with NF1 extending through 4 generations (grandfather, mother, 2 uncles, 1 aunt and her only 14-year-old son affected). Both patients had the common ALL cell phenotype, a normal karyotype and very aggressive clinical evolution.

Table 1. Clinical characteristics of the patients.

	Case #1	*Case #2*
Age at diagnosis of ALL/sex	19/M	31/F
Familial history of neurofibromatosis (affected relatives)	Mother Sister Uncles (2) Aunt Son	Grandfather Mother
Clinical features of neurofibromatosis:		
>6 café-au-lait spots	+	+
Cutaneous neurofibromas	+	+
Freckles	+	+
Other abnormalities	-	-
ALL phenotype	Common	Common
Karyotype	Normal	Normal

Because NF1 in one of the most common autosomal dominant disorders (with an incidence of 1:3000 neonates), one must be aware that the association observed could easily occur by chance. Although case reports are not appropriate for causation assessment, they have the potential to generate new knowledge by stimulating reporting of additional single cases or the development of more traditional epidemiologic surveys that may lead to a precise estimate of risk. For additional information about the molecular pathoegensis of acute lymphoblastic leukemia, the reader is referred to recent papers in this journal.[9,10]

Thus, besides the well known association between NF1 and myeloid disorders in children, our two case reports reveal a possible causal relation between familial NF1 and adult onset ALL.

Antoni Julia, Jaume Ayguasanosa, Antonio Blanco

Servei d'Hematologia, Vall d'Hebron Hospitals, Barcelona, Spain

Key words

Neurofibromatosis, ALL

Correspondence

Antoni Julia, M.D., Servei d´Hematologia, Vall d'Hebron Hospitals, 0835 Barcelona, Spain. Fax: international +34-93-2746015 – E-mail: tjulia@hg.vhebron.es

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Central nervous system involvement in acute promyelocytic leukemia. A description of two cases and review of the literature

Sir,

Extramedullary involvement is infrequent in acute promyelocytic leukemia (APL), but it has been suggested that its incidence might be increasing. Two patients with APL and central nervous system (CNS) involvement are described and the possible relationship between this complication and new treatment approaches of APL are discussed.

Case #1. A 45-year-old woman was diagnosed with APL. Cytogenetic studies revealed a complex karyotype in bone marrow (46,XX,r(9) t(17;15;10) (q11; q22;q24) and a bcr-1 pattern of the PML-RARα fusion protein. A molecular complete remission (CR) was achieved within the first month from starting ATRA and standard chemotherapy. Five days after administration of a consolidation course she presented with a third cranial nerve palsy and examination of the cerebrospinal fluid revealed the presence of promyelocytes in which the PML-RARα rearrangement was detected. She was given ATRA plus intrathecal chemotherapy without achieving a response and died shortly thereafter from progressive disease.

Case #2. A 45-year-old man was diagnosed with APL and a bcr-1 pattern of the PML-RARα fusion protein was demonstrated in peripheral blood. CR was achieved after treatment with ATRA and standard chemotherapy. Twenty months later he presented with a bone marrow relapse. After attaining a second CR he underwent an allogeneic bone marrow transplantation (BMT). Twenty months after the BMT he developed a spinal cord syndrome due to a paravertebral mass. Biopsy of the tumor revealed promyelocytes with a PML-RARα rearrangement (bcr-1). Despite treatment with ATRA and local radiotherapy, the patient died from disease progression.

Extramedullary involvement, including CNS infiltration, has been rarely reported in APL. It usually occurs

Table 1. Reported cases of extramedullary disease in APL.

Author	*Year*	*A/S*	*ATRA**	*Extramedullary site*	*Interval*	*Cyto*	*Outcome*
Bermengo	1975	82/M	No	Cutaneous	0m	NA	Death (PD)
Belasco	1978	9m/M	No	Soft tissue	0m	NA	CR
Nihei	1984	55/M	No	Mediastinum and muscle	4m	NA	Death (PD)
Kubonishi	1984	23/M	No	Mediastinum	0m	NA	Death (PD)
Kanakura	1987	44/F	No	Intracerebral mass, CSF	0m	NA	CR (23m)
Baer	1989	59/F	No	Cutaneous	36m	NA	BM relapse
Zuiable	1989	31/M	No	SNC (extradural mass)	0m	No	BM relapse
Rush	1990	13m/M	No	Mandibular	22m	No	CR (16m)
Ajarim	1990	21/M	No	Mediastinum (also BM)	0m	No	BM relapse
Niazi	1991	26/M	No	Cutaneous and CNS	NA	NA	Death (PD)
Brown	1992	37/F	No	Optic nerve (also BM)	22m	Yes	CR (NA)
Longacre	1993	19/M	NA	Cutaneous	NA	NA	NA
Thomas	1994	68/F	Yes	Subcutaneous	10m	Yes	CR (8m)
Weiss	1994	31/M	Yes	External auditory canal	11m	Yes	CR (NA)
Weiss	1994	33/M	Yes	Cutaneous &lymph node	11m	Yes	CR (NA)
Giralt	1994	23/M	Yes	Cutaneous & CSF	13m	Yes	CR (NA)
Giralt	1994	35/M	Yes	Cutaneous	1,5m	Yes	Death (PD)
Giralt	1994	47/F	Yes	Cutaneous	5m	NA	Death (PD)
Tosi	1995	27/M	No	Epidural	0m	Yes	CR (14m)
Hazneradoglu	1995	19/M	No	Gingiva	15m	NA	Death (PD)
Békássy	1995	24/F	NA	Cutaneous	NA	NA	Response
Békássy	1995	3/F	NA	Spinal	NA	NA	Death (PD)
Békássy	1995	26/M	NA	Testicle&spinal	NA	NA	Death (toxic)
Lederman	1995	46/F	No	Subcutaneous&CNS	6m	Yes	Response
Selleri	1996	31/F	No	Cutaneous	18m	Yes	CR (25m)
Wiernik	1996	5/F	Yes	Gingiva	21m	Yes	Death (PD)
Wiernik	1996	25/F	No	Cutaneous (also BM)	6m	Yes	Death (PD)
Chen	1996	74/M	No	Spleen	0m	Yes	Death (PD)
Martinelli	1997	42/M	No	Bone (L4)	0m	Yes	BM relapse
Evans	1997	23/M	Yes	CNS	15m	Yes	Death (GVHD)
Evans	1997	22/M	Yes	Bone (mastoid)	6m	Yes	CR (NA)
Evans	1997	49/F	Yes	CNS(CSF+)	NA	Yes	Death (PD)
Molero	1997	40/M	Yes	CNS(CSF+)	13m	Yes	Death (PD)
Ueda	1997	66/M	Yes	Cutaneous (also BM)	7m	Yes	NA
Del Río	1997	28/F	No	Cutaneous (also BM)	7m	NA	PD
Kishimoto	1997	11/F	Yes	Cutaneous	2-3m	Yes	PD
Castagnola	1997	23/M	No	CNS	11m	Yes	Death (PD)
Maloisel	1997	42/M	Yes	Colon	NA	Yes	Death(PD)
Forrest	1997	34/M	Yes	Testicle	120m	Yes	Death (PD)
Liso	1998	17/M	No	Lymph node	25m	Yes	Death (PD)
Liso	1998	48/M	No	Cutaneous	14m	Yes	Death (PD)
Liso	1998	22/F	No	Cutaneous	14m	Yes	Death (GVHD)
Liso	1998	25/F	No	Cutaneous	12m	Yes	Death (PD)
Liso	1998	30/M	Yes	CNS (CSF+)	9m	Yes	CR (12m)
Liso	1998	44/F	Yes	Cutaneous	10m	Yes	CR (20m)
Liso	1998	45/M	Yes	Cutaneous	0m	Yes	CR (31m)
Present case		37/F	Yes	CNS (CSF+)	1,5m	Yes	Death (PD)
Present case		45/M	Yes	CNS (extradural mass)	31m	Yes	Death (PD)

A/S: age and sex; ATRA: prior therapy with ATRA; Interval: months from diagnosis to extramedullary disease; Cyto: t(15;17)/PML-RARα fusion protein. BM: bone marrow; CSF: cerebrospinal fluid; PD: progressive disease; NA: not available.

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shortly before or concomitantly to a bone marrow relapse, although in some cases it is apparent at the diagnosis or late in the course of the disease, up to ten years from the initial diagnosis. The skin or the CNS are the sites most frequently involved. Although the prognostic significance of extramedullary involvement in APL has not been formally assessed, from a review of the literature (see Table 1), it appears that about one third of patients may achieve a complete and in some cases sustained remission of the disease. Whether the incidence of this complication is increasing is a matter of debate, as it is its potential relationship with ATRA therapy.[1-4] A number of reasons could account for the increased incidence of extramedullary involvement. Firstly, the longer survival of patients treated with ATRA would increase the number of patients at risk of developing this type of relapse. Secondly, *in vitro* studies have shown that ATRA modulates the expression of adhesion molecules in APL cells enhancing their adhesiveness and motility.[5,6] These mechanisms might explain the efflux of leukemic cells from the bone marrow to the tissues in the ATRA syndrome and might also play a role in extramedullary relapses after ATRA treatment. Nevertheless, extramedullary APL may develop after chemotherapy or at presentation. In conclusion, although rare, extramedullary involvement is possible in patients with APL, a fact that should be considered in the management of these patients. Finally, the actual incidence of this complication and its relationship to new therapies should be prospectively assessed.[7-10]

Susana Rives, Mireia Camós, Francesc Bosch, Jordi Esteve, Neus Villamor, Emili Montserrat

Department of Hematology and Postgraduate School of Hematology 'Farreras Valenti', Department of Medicine, Institut d'Investigacions Biomèdiques August Pí i Sunyer (IDIBAPS), Hospital Clínic, University of Barcelona, Spain

Key words

Acute promyelocytic leukemia, ATRA, extramedullary involvement, CNS involvement

Acknowledgments

This work was supported in part by grants 1997 SGR 00211 and Maderas Llodio, S.A.L.M.C.

Correspondence

E. Montserrat, M.D., Department of Hematology, Hospital Clínic, University of Barcelona, Villarroel 170, 08036 Barcelona, Spain. Phone/Fax: +34-93-2275475 – E-mail: emili@medicina.ub.es

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Legionella sp pneumonia in patients with hematologic diseases. A study of 10 episodes from a series of 67 cases of pneumonia

Sir,

Legionella pneumophila is a significant pathogen for immunocompromised patients, especially for those with impaired cell-mediated immunity.[1,2] In spite of the fact that patients with malignant hematologic diseases frequently have neutropenia and/or immunosuppression and usually receive glucocorticoids as cytotoxic drugs, information about the prevalence and evolution of pneumonias by *Legionella sp* in these patients is scarce.[2] We summarize the presenting features and response to treatment of 9 patients with hematologic diseases who developed 10 episodes of *Legionella* pneumonia diagnosed in a single institution over a 2.5-year period.

A study of all cases of pneumonia diagnosed in a hematology unit from January 1995 to June 1998 was carried out. One hundred and twenty-seven episodes of pneumonia in 106 patients were diagnosed, 68 were community-acquired and 59 nosocomial. In 67 cases radioimmunoassay for *Legionella pneumophila* serogroup 1 (LPS1) antigen in urine was performed, being positive in 10 (one patient had two episodes of pneumonia). In two cases, *Legionella* was also identified in the culture of bronchoalveolar lavage (performed in 15 cases of pneumonia). In the present study, *Legionella pneumophila* was the most frequently found micro-organism (10 cases, 15%), followed by *Streptococcus pneumoniae* (9 cases, 13%) and *Pseudo-*

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Table 1. Clinical features of the ten episodes of *Legionella pneumonia*.

Patient	Age (years)	Hematologic disease	Immunosuppressive drug	Neutropenia*	Acquisition of pneumonia	Respiratory failure	Erythromycin	Death
1	84	NHL	None	No	Nosocomial	Yes	Yes	No
2	26	NHL	Dexamethasone	No	Nosocomial	No	Yes	No
2°	26	NHL	Dexamethasone	No	Nosocomial	No	Yes	No
3	57	NHL	Methyl-prednisolone	No	Nosocomial	No	Yes	No
4	75	ATP	Methyl-prednisolone	No	Nosocomial	No	Yes	No
5	63	NHL	Methyl-prednisolone	No	Nosocomial	Yes	Yes	No
6	16	AML	None	Yes	Community	Yes	Yes	No
7	83	SAA	Methyl-prednisolone	Yes	Community	Yes	No	Yes
8	35	ALL	None	Yes	Nosocomial	No	Yes	No
9	52	ALL	Methyl-prednisolone	Yes	Nosocomial	Yes	Yes	No

*°Recurrent pneumonia; *granulocyte count of < 1×10^9/L; NHL: non-Hodgkin's lymphoma; ATP: acute thrombocytopenic purpura; AML: acute myelogenous leukemia; SAA: severe aplastic anemia; ALL: acute lymphocytic leukemia.*

monas aeruginosa (5 cases, 7%). Table 1 summarizes the main clinical characteristics associated with the 10 episodes of *Legionella* pneumonia. The median age of the patients was 57 years (range 16-84). Eight of the ten episodes of pneumonia were nosocomial. Fever, cough and dyspnea occurred in all patients and five complained of chest pain. Pneumonia was bilateral in two cases. Five patients developed respiratory failure but none required mechanical ventilation. The median time of disappearance of fever after the initiation of erythromycin (1 g/6 hours i.v.) was 96 hours (range 24-168). The median number of days of treatment with erythromycin was 21 (range 15-90). Recurrence of *Legionella* pneumonia was seen in one patient with lymphoma treated with dexamethasone for a long time. In this case, the pneumonia was cured with ofloxacin (400 mg/12 hours p.o.) for six weeks. Only one patient died.

The high prevalence of *Legionella* pneumonia found in this series can be explained by the fact that legionellosis is a prevalent nosocomial infection in our hospital,[3,4] despite several attempts at eradication (heating and hyperchloration of water). Although there are more than 14 serogroups of *Legionella pneumophila*, the predominant ones are 1, 4 and 6. Urinary antigen detection of LPS1 by ELISA is a good diagnostic tool, with a specificity of 100% and sensitivity of between 70 and 100%.[5,6]

All our patients were immunocompromised. Chemotherapy, treatment with steroids and other situations of immunosuppression such as organ transplants predispose to this infection, suggesting that cell-mediated immunity is the most important defensive mechanism against *Legionella*.[1,2,7-9] The evolution of *Legionella* pneumonia is worse in these patients, mainly depending on the setting in which the pneumonia is acquired (community or nosocomial), the virulence of the *Legionella sp* and the prompt initiation of treatment with erythromycin. All our patients except one received erythromycin at the time of the diagnosis of pneumonia, which probably explains the good evolution of all but one of the cases.

Recurrent *Legionella* pneumonia in patients treated with erythromycin for less than three weeks has been reported in chronically ill and immunocompromised hosts.[1,9] *Legionella* may survive for weeks within alveolar macrophages if an effective cellular immune response is absent. The best treatment for recurrent *Legionella* pneumonia, therefore, probably includes drugs that kill intracellular bacteria or inhibit their growth for prolonged periods of time such as azithromycin or fluoroquinolones,[10] which we gave to one patient in our series.

Francesc Fernández-Avilés, Montserrat Batlle, Josep-Maria Ribera, Lourdes Matas, Miguel Sabrià,° Evarist Feliu*

*Departments of Hematology, *Microbiology and °Internal Medicine, Hospital Universitari Germans Trias i Pujol, Badalona, Universitat Autònoma de Barcelona, Spain*

Key words

Legionella sp, pneumonia, nosocomial, hematologic diseases, immunosuppression

Acknowledgments

Supported in part by grant 97/1049 from Fondo de Investigaciones Sanitarias and grant 98-P-EF from José Carreras International Leukemia Foundation.

Correspondence

Josep-Maria Ribera Santasusana, MD, PhD., Servicio de Hematología-Hemoterapia, Hospital Universitari Germans Trias i Pujol, Carretera de Canyet s/n, 08916 Badalona, Spain. Phone: international +34-93-4978868 – Fax: international +34-93-4978843 – E-mail: jmribera@ns.hugtip.scs.es

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Prediction of blood cyclosporine concentrations in non-obese and obese hematologic patients with multidrug resistance using total, lean and different adipose factor dosing body weights

Sir,

Cyclosporine (CsA) is a highly lipophilic cyclic polypeptide drug,[1] thus better predictions of blood CsA concentrations would be expected from using *total body weight* (TBW) rather than *lean body weight* (LBW) or *adipose factor dosing body weight* (AFDBW). However, several studies show that CsA distribution correlates better with LBW in obese patients and suggest that CsA steady-state concentrations mainly depend on LBW.[2,3] This leads to difficulty in choosing which body weight to use to optimize CsA dosage regimens and predict blood CsA concentrations in non-obese and obese patients.

Thirteen female and twenty-eight male hematologic patients with multidrug resistance were treated by continuous intravenous CsA infusion (Table 1). Blood CsA concentrations were monitored about 4 times a day during infusion and 11 times after infusion (0, 0.5, 1, 2, 3, 5, 7, 9, 12, 24, and 36 hours after infusion), and were immediately analyzed using a fluorescence polarization immunoassay method (TDx, Abbott Laboratories, Diagnostic Division, Irving, TX, USA).[5]

The PKS program (Abbottbase Pharmacokinetic System, version 1.10, Abbott Laboratories, IL, USA, 1992) was used to predict blood CsA concentration using LBW, 25% AFDBW, 50% AFDBW, 75% AFDBW and TBW with a two-compartment model with volume of distribution in the central compartment (V_c=0.70±0.26 L/kg), clearance (CL=0.25±0.08 L/h/kg) and intercompartment rate constants (k_{12}=0.52±0.31 and k_{21}=0.07±0.02/h).[6,7] LBW = –111.621 + (3.636× height in inches) for adult females and LBW = –130.736 + (4.064×height in inches) for adult males. Dosing body weight = LBW + adipose factor × (TBW – LBW)/100, where adipose factor is set at 25%, 50% and 75%, respectively.

The measured and predicted concentrations were used to calculate percentage prediction errors [100× (predicted concentration – measured concentration)/ (measured concentration)][8] and absolute/relative performances.[9]

Blood CsA concentrations were divided into pre-steady-state, steady-state (infusion rate/clearance)[10] and post-steady-state. Table 2 shows the percentage prediction errors. The Friedman ANOVA test indicates that the medians among five dosing body weights at each kinetic state are not equal at p<0.001

Table 1. Patients demographics and CsA dosage.

Patients	*Obese situation*	*Numbers*	*Age (years)*	*Height (cm)*	*TBW (kg)*	*LBW (kg)*	*Obesity index (kg/m²)*	*TBW Dose (mg/kg/day)*	*LBW Dose (mg/kg/day)*	*IVT (days)*
Female	non-obese	6	60±6	165±7	64±9	56.6±4.9	23.4±1.2	9.6±2.7	10.8±2.8	3.7±1.0
	moderately obese	7	58±4	160±3	67±5	53.3±2.0	26.1±1.0	9.9±1.2	12.4±1.4	3.6±1.2
Male	non-obese	12	38±15	172±7	70±5	66.7±5.2	23.7±0.7	11.3±1.8	12.0±2.1	3.4±1.2
	moderately obese	14	44±12	172±3	78±5	65.7±2.2	26.5±1.5	10.4±2.3	12.3±2.6	4.2±0.4
	seriously obese	2	51±17	178±11	103±19	69.7±7.7	32.3±2.2	10.5±0.9	15.4±2.4	2.3±2.2

The data are expressed as mean±SD. Non-obese, moderately obese and seriously obese are defined as obesity indices <25 kg/m², 25-29.9 kg/m² and 30-39.9 kg/m², respectively.[4] IVT, the duration of the continuous intravenous infusion. The average time interval between two courses is 77±73 days (mean±SD). Statistical differences were found between TBW and LBW, between TBW and LBW doses at p<0.05 level (the paired Student's t-test). TBW and LBW doses were calculated by dividing the daily dose by TBW and LBW, respectively.

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Table 2. Percentage prediction errors.

Gender	Obese situation	kinetic state	n	TBW	75%AFDBW	50%AFDBW	25%AFDBW	LBW
Female	Non-obese	pre-steady state	10	-15.8 (-39.6 – -5.0)	-14.1 (-37.2 – -3.3)	-12.3 (-34.5 – -1.5)	-10.4 (-32.5 – 0.3)	-8.5 (-30.4 – 2.3)
		steady-state	70	19.3 (-20.6 – 37.2)	22.7 (-18.6 – 40.0)	27.0 (-16.5 – 42.3)	32.6 (-14.1 – 44.7)	34.9 (-10.2 – 48.3)
		post-steady state	62	12.9 (-33.7 – 64.9)	15.7 (-32.8 – 66.9)	17.0 (-32.0 – 68.6)	18.3 (-31.1 – 73.6)	21.5 (-30.2-79.6)
	Moderately obese	pre-steady state	15	-22.2 (-28.6 – -13.8)	-17.6 (-24.9 – -9.5)	-12.4 (-20.9 – -4.9)	-6.5 (-16.5 – 0.3)	0.3 (-11.5 – 6.9)
		steady-state	89	22.1 (10.9 – 45.3)	28.8 (15.9 – 52.8)	34.9 (21.89 – 63.1)	42.4 (29.7 – 74.0)	50.8 (38.0 – 86.7)
		post-steady state	65	28.1 (7.3 – 54.9)	33.9 (12.5 – 64.1)	40.3 (18.3 – 74.4)	47.3 (24.5 – 86.2)	55.5 (31.0 – 99.7)
Male	Non-obese	pre-steady state	29	-9.0 (-27.9 – -0.4)	-6.8 (-26.9 – 1.8)	-5.6 (-26.0 – 4.0)	-3.5 (-24.9 – 6.4)	-2.9 (-23.9 – 9.9)
		steady-state	169	27.8 (9.2 – 45.4)	29.6 (10.9 – 48.2)	32.2 (12.5 – 50.9)	34.5 (14.6 – 53.2)	36.4 (16.5 – 55.5)
		post-steady state	122	35.9 (13.1 – 60.3)	38.0 (15.2 – 62.9)	40.0 (17.5 – 65.4)	41.4 (19.2 – 67.4)	43.6 (20.5 – 70.3)
	Moderately obese	pre-steady state	30	-28.2 (-60.4 – -16.4)	-25.2 (-57.9 – -12.2)	-21.9 (-55 – -8.3)	-18.3 (-54.1 – -4.1)	-14.4 (-52.4 – 0.5)
		steady-state	167	-6.5 (-36.2 – 24.8)	-2.8 (-34.1 – 29.1)	1.5 (-29.7 – 33.8)	5.1 (-25.7 – 39.0)	9.8 (-21.3 – 45.9)
		post-steady state	143	6.7 (-38.1 – 53.5)	11.1 (-34.3 – 60.3)	14.7 (-29.9 – 69.8)	22.9 (-25.9 – 78.8)	28.5 (-23.6 – 89.4)
	Seriously obese	pre-steady state	3	-37.7 (-39.5 – -18.6)	-33.2 (-35.0 – -11.3)	-27.8 (-29.8 – 2.7)	-21.5 (-23.6 – 7.8)	-13.9 (-16.2 – 20.9)
		steady-state	14	3.9 (-16.6 – 19.5)	12.8 (-10.4 – 28.5)	23.4 (-3.1 – 38.9)	36.1 (5.4 – 51.1)	49.2 (15.6 – 68.1)
		post-steady state	19	49.9 (16.4 – 74.4)	61.1 (27.7 – 87.5)	76.1 (41.3 – 103.3)	97.2 (58.3 – 127.7)	120.0 (78.2 – 153.8)

Data are presented as the median with interquartile range. n, number of comparisons.

level. The absolute and relative predictive performances show the similar results as percentage prediction errors.

The results suggest using TBW to predict steady-state and post-steady-state blood CsA concentrations and LBW to predict pre-steady-state blood CsA concentrations. The results indirectly support the concept that CsA distribution obeys physicochemical principles.[1]

The estimation of pharmacokinetic parameters using TBW might lead to favorable TBW predictions. The facts that at pre-steady-state CsA might not fully distribute into fatty tissues and CsA concentration might not be dominated by CsA metabolism might lead to favorable LBW predictions.

The time of the continuous intravenous infusion was not identical in each patient, because several patients felt uncomfortable during infusion. Although the ages were not comparable between females and males, we did not consider this problematic because we were mainly concerned with inter-method comparisons at each age group. However, we hope to find patients at different ages in future studies, so we can analyze the integrity of CsA metabolism, which decreases with age, better. We also hope to find seriously obese female patients in future studies.

Guang Wu, Mario Furlanut

Institute of Clinical Pharmacology and Toxicology, Chair of Special Pharmacology, Medical School, University of Udine, Italy

Acknowledgments

The PKS program was a kind gift from Abbott Laboratories to Prof. Mario Furlanut.

Key words

Cyclosporine, dosing body weight, hematologic patient with multidrug resistance, inter-method comparison, lean body weight, obesity, prediction, total body weight.

Correspondence

Guang Wu, M.D. PhD., Istituto di Farmacologia Clinica e Tossicologia, Cattedra di Farmacologia Speciale, Facoltà di Medicina e Chirurgia, Università degli Studi di Udine, p.le Santa Maria della Misericordia, 33100 Udine, Italy. Phone: international +39-0432-559833 – Fax: international +39-0432-559833.

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Indolent lymphoproliferative disease of large granular lymphocytes after lung transplantation

Sir,

Sequential assessment of peripheral blood lymphocyte subsets, useful for following post-transplantation immune reconstitution and detecting infectious or rejection episodes, also allows identification of lymphoproliferative disorders or unusual patterns in some patients.[1] We report the case of a lung transplant recipient who has had, for over 7 years, a persistent immunophenotypic pattern reminiscent of that reported in LGL leukemia and lymphoproliferative diseases of granular lymphocytes (LDGL).

DR, 55 years old, suffered from chronic respiratory insufficiency due to panacinar emphysema with α1-antitrypsin deficiency, which led to a single lung transplant in February 1991. Post-transplant induction immunosuppression was achieved by anti-lymphocyte globulins (ALG; Thymoglobulin, Merieux, Lyon, France), followed by a maintenance regimen of cyclosporine, azathioprine and corticosteroids.

Three episodes of grade I acute rejection were treated by reinforced corticosteroids. Two episodes of bronchiolitis obliterans were controlled by reinforced immunosuppression including ALG treatment in the second instance. Pulmonary function tests have remained stable since.

Herpetic bronchitis was treated by acyclovir. CMV seroconversion, observed concomitantly to the detection of CMV cellular inclusions in a transbronchial biopsy, was treated by ganciclovir. Later, a CMV and *Pneumocystis carinii* lung infection developed which prompted treatment with ganciclovir and sulfamethoxazole-trimethoprim. Antibiotic therapy was necessary to clear several infectious episodes due to *Haemophilus influenzae*, *Aspergillus fumigatus* and *Pseudomonas aeruginosa*. Two epidermoid carcinomas developed in 1996 and in 1998; they were resolved after surgery and chemotherapy.

Sequential immunophenotyping of PBL subsets was performed in the same Immunology laboratory at regular intervals over 7 years (Figure 1) by flow cytometry (Coulter Corporation, Hialeah, FL, USA; reagents from Coulter Corporation and Immunotech, Marseille, France). Post-transplantation and during immunosuppressive treatment, there was a progressive restoration of the different subsets which had collapsed after ALG treatment. From September 1992 on, however, the level of T-cell subsets decreased severely to around 10%, i.e. absolute numbers of $0.11\pm0.05\times10^9$/L, associated with persistently high levels of CD57$^+$ cells (mean overall level out of ALG treatment 60.5±19%, $0.86\pm0.39\times10^9$/L). White blood cell and lymphocyte counts remained within normal ranges, there being steady levels of around 4×10^9/L polymorphonuclear cells. Cytologic examination consistently showed over 60% of typical large granular lymphocytes.

Extensive immunophenotypic studies demonstrated that the predominant population of CD57$^+$ lymphocytes expressed surface CD7, CD2 and cytoplasmic CD3, but lacked CD5, CD3, CD4, CD8 and the T-cell receptor. CD16 and CD94 were always present but CD56 expression was only observed occasional-

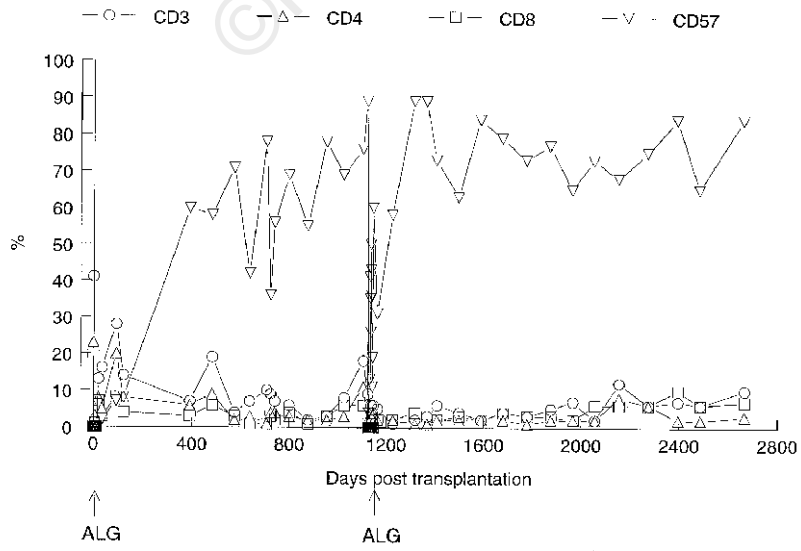

Figure 1. Follow-up of CD3$^+$, CD4$^+$, CD8$^+$ and CD57$^+$ peripheral blood lymphocyte subsets over 7 years following lung transplantation.
ALG: anti-lymphocyte globulin treatments.

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ly. CD158a and b were not expressed by these cells. Natural killer activity was normal.

This case report describes an unusual context for the development of persistent NK lymphocytosis, i.e. more than one year post-transplantation, after infectious episodes not unusual in transplantation. This patient differs from the 26 $CD8^-/CD4^-/NKa^+$ cases reported by Scott *et al.*,[2] since nearly all lymphocytes were LGL, yet the absolute counts were lower than 4.5×10^9/L. NK-LGL leukemia[3] can also be ruled out in the absence of neutropenia, visceral involvement or coagulopathy. The long term and indolent character of this immunohematologic rarity is more reminiscent of the chronic NK cell lymphocytoses described after infectious episodes by several authors,[4,5] and given diagnosis criteria by Semenzato *et al.*[6] According to the latter, the patient described here appears to be another case of the very rare post infectious low count $CD3^-$ LDGL, only observed in 2 out of 195 patients by those authors. The indolent evolution of this patient's disease could be related to the immunosuppression he receives as rejection prevention, which matches attempted therapeutic approaches in NK lymphocytosis.[3]

Marie N Kolopp-Sarda, François Chabot,° Rachel Petermann-Khder,* Anne M Mattei,# Gilbert C. Faure,* Marie C. Béné**

**Laboratoire d'Immunologie; °Maladies Respiratoires; #Chirurgie Cardiaque et Transplantation, CHU & Faculté de Médecine de Nancy, Vandoeuvre les Nancy, France*

Key words

Lung transplantation, lymphoproliferative diseases

Correspondence

Prof. Marie Christine Béné, Laboratoire d'Immunologie du CHU, BP184, 54500 Vandoeuvre les Nancy, France. Phone: international +33-383-592856 – Fax: international +33-383-446022 – E-mail: bene@grip.u-nancy.fr

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Colorectal cancer and HFE gene mutations

Sir,

Hereditary hemochromatosis (HH) is characterized by an increased absorption of iron resulting in excess deposition of this metal in parenchymal cells of the liver, heart, and certain endocrine organs.[1-3] Patients with HH have an increased risk, in relation to their increased iron stores, of suffering liver and esophageal cancer and skin melanoma.[4] The relative risk of subjects with moderately high levels of serum transferrin saturation and high serum ferritin (laboratory abnormalities similar to those found in HH heterozygotes) suffering from colorectal cancer is three times higher than in the normal population.[5,6]

Whether HH heterozygotes have a higher incidence of colorectal cancer is not known, although a slightly higher RR (1.28) in these subjects was found in one study.[7]

In 1996 Feder *et al.*[8] identified a gene strongly linked to HH, which is now known as HFE. A change in a single base pair of this gene (C282Y) is clearly associated with HH, and subjects who share a normal haplotype with C282Y are considered heterozygotes for the disease.[9] The relationship between a second genomic change (H63D) and HH is currently unclear. We investigated both substitutions in 116 patients with colorectal cancer and in 108 healthy subjects in order to compare the frequencies of the substitutions and determine whether there is higher than expected proportion of HH heterozygotes in patients with colorectal cancer.

A total of 116 DNA samples which had been stored at 4°C were thawed from a colorectal cancer DNA bank. DNA samples from 108 healthy blood donors were used as normal controls. The distribution of sexes was similar in both groups (54.3% males in the cancer group, 57.4% males in the control group), but that of age was heterogeneous (mean age 66.9 years in cases vs 40 in controls, $p<0.05$). C282Y and H63D mutations were screened for by using enzymatic digestion of PCR products encompassing the mutation sites as described elsewhere.[10] The frequencies of mutations in

Table 1. Genotype frequencies of mutations in the HFE gene in patients with colorectal cancer anf healthy controls.

Genotypes	*Cases n=116*	*Controls n=108*
HH/CC	68	70
HH/CY	5	6
HD/CC	36	28
DD/CC	6	2
HD/CY	1	2
C282Y*	2.6 (1-5.5)	3.7 (1.6-7.2)
H63D*	21.1 (16.1-26.9)	15.7 (11.2-21.3)

**Allelic frequencies (%; 95%CI). Genotypes are given for aminoacid 63 (H63D)/aminoacid 282 (C282Y) of protein. CC/HH corresponds to the wild type.*

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both groups were compared using Fisher's exact test.

The genotypic frequencies for mutations of the HFE gene are shown in Table 1. The frequencies in cases and controls were similar to those found in previously published studies. The observed differences were not statistically significant. We found no homozygotes for C282Y (HH patient) in either group. These results rule out the existence of a strong association (OR ≥3) between HFE mutations and colorectal cancer. Patients with colorectal cancer do not appear to have a higher prevalence of HH heterozygosity than normal blood donors. A criticism to this study is that the groups were heterogeneous for age, and it is possible that some controls may develop colorectal cancer in the future, but this subgroup will be very small with little influence on the overall results. Our study suggests that the epidemiologic relationship between colorectal cancer and increased body iron is probably more the result of dietary and environmental factors than genetic factors. New epidemiologic studies specifically designed to prove this environmental relationship will be necessary to shed further light onto these observations.

Albert Altés, Enric Gimferrer,° Gabriel Capella,# M. Jesús Barceló,* Montserrat Baiget**

**Hematology Unit, Clinical Laboratory, Hospital Esperit Sant, Santa Coloma de Gramenet; °Ferropathology and Radicalosis Unit, #Gastrointestinal Research Laboratory, Departament of Genetics, Sant Pau Hospital, Barcelona, Spain*

Keywords

Hereditary hemochromatosis, HFE, colorectal cancer, iron.

Correspondence

Albert Altés MD, Hematology Division, Hospital de Sant Pau, Avda. S.A.M. Claret 167, 08025 Barcelona, Spain. Phone: international +34-93-2919396 – Fax: international +34-93-2919466 – E-mail: aaltesh@hsp.santpau.es

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