Preclinical evaluation of a bone marrow autograft culture procedure for generating lymphokine-activated killer cells in vitro

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H-G KLINGEMANN, H DEAL, D REID, CJ EAVES. Preclinical evaluation of a bone marrow autograft culture procedure for generating lymphokine-activated killer cells in vitro. Can J Infect Dis 1992;3(Suppl B):123B-127B. Despite the use of high dose chemoradiotherapy for the treatment of acute leukemia, relapse continues to be a major cause of death in patients given an autologous bone marrow transplant. Further augmentation of pretransplant chemotherapy causes life threatening toxicity to nonhematopoietic tissues and the effectiveness of currently available in vivo purging methods in reducing the relapse rate is unclear. Recently, data from experimental models have suggested that bone marrow-derived lymphokine (IL-2)-activated killer (BM-LAK) cells might be used to eliminate residual leukemic cells both in vivo and in vitro.

To evaluate this possibility clinically, a procedure was developed for culturing whole marrow harvests with IL-2 prior to use as autografts, and a number of variables examined that might affect either the generation of BM-LAK cells or the recovery of the primitive hematopoietic cells. The use of Dexter long term culture (LTC) conditions, which expose the cells to horse serum and hydrocortisone, supported LAK cell generation as effectively as fetal calf serum (FCS)-containing medium in seven-day cultures. Maintenance of BM-LAK cell activity after a further seven days of culture in the presence of IL-2 was also tested. As in the clinical setting, patients would receive IL-2 in vivo for an additional week immediately following infusion of the cultured marrow autograft. Generation of BM-LAK activity was dependent on the presence of IL-2 and could be sustained by further incubation in medium containing IL-2. Primitive hematopoietic cells were quantitated by measuring the number of in vitro colony-forming progenitors produced after five weeks in secondary Dexter-type LTC. Maintenance of these 'LTC-initiating cells' was unaffected by IL-2 in the culture medium. These results suggest that LAK cells can be generated efficiently in seven-day marrow autograft cultures containing IL-2 under conditions that allow the most primitive human hematopoietic cells currently detectable to be maintained. (Pour résumé, voir page 124B)

Key Words: Bone marrow transplantation, Interleukin-2, Leukemia, Residual disease
Évaluation préclinique d'une technique de culture d'autogreffe de moelle osseuse pour la production de cellules tueuses activées aux lymphokines in vitro

RÉSUMÉ: En dépit de l'utilisation de doses élevées de chimioradiothérapie pour le traitement de la leucémie aiguë, les récurrences continuent d'être une cause importante de décès chez les patients qui reçoivent une transplantation autologue de moelle osseuse. Le recours plus fréquent à la chimioradiothérapie avant la transplantation peut induire une toxicité potentiellement létale pour les tissus non hématopoïétiques. De plus l'efficacité des méthodes ex vivo de purge actuellement disponibles pour réduire le taux de récurrence demeure imprécise. Récemment, des résultats tirés de modèles expérimentaux donnent à penser que les cellules tueuses activées aux lymphokines (IL-2) dérivées de moelle osseuse, pourraient être utilisées pour éliminer les cellules leucémiques résiduelles tant in vivo que in vitro. Afin d'évaluer cette possibilité, une technique a été mise au point pour cultiver la moelle avec IL-2 avant de l'utiliser en autogreffe. Un certain nombre de variables ont été examinées pour connaître leur influence sur la production de cellules BM-LAK ou la récupération de cellules primitives hématopoïétiques. L'utilisation de conditions de culture à long terme Dexter (LTC) qui exposent les cellules à du sérum équin et de l'hydrocortisone a favorisé la production de cellules LAK aussi efficacement qu'un milieu de culture renfermant du sérum de foetus de veau sur 7 jours. Le maintien de l'activité des cellules BM-LAK pendant une autre période de 7 jours de culture en présence d'IL-2 a également été soumis à l'épreuve puisque dans cette étude clinique, les patients devaient recevoir IL-2 in vivo pour une autre semaine immédiatement après la perfusion de l'autogreffe de moelle cultivée. La production de l'activité BM-LAK s'est révélée dépendante de la présence d'IL-2 et pouvait être maintenue par une incubation prolongée dans le milieu de culture qui renfermait de l'IL-2. Les cellules primitives hématopoïétiques ont été quantifiées par la mesure du nombre de progéniteurs formant des colonies in vitro après 5 semaines dans un LTC secondaire de type Dexter. Le maintien de ces cellules de culture LTC n'a pas été affecté par IL-2 dans le milieu de culture. Ces résultats suggèrent que les cellules LAK peuvent être générées efficacement dans des cultures d'autogreffes de moelle sur 7 jours renfermant IL-2, dans des conditions qui permettent le maintien des cellules hématopoïétiques humaines primitives présentement décélables.

Bone marrow transplantation has emerged as a curative treatment for a number of hematological malignancies including acute and chronic leukemia, malignant lymphoma and myeloma. In protocols that rely on the use of marrow autografts, the patient's bone marrow is harvested and reinfused after delivery of high dose chemoradiotherapy. The use of strategies for rescuing the marrow with autologous cells has many attractions but potentially is limited by the possibility of contaminating malignant cells in the transplant even if the cells have been collected in remission and some form of malignant cell purging has been performed. Allogeneic bone marrow transplantation from a tissue-matched sibling or unrelated donor provides bone marrow free from malignant cells; however, even though the recipient and donor are matched for major histocompatibility complex (MHC) antigens, minor non-MHC antigens exist for which no predictive testing is available, and these can result in fatal graft-versus-host disease (GVHD) in a proportion of recipients (1).

GVHD is mediated by immunologically activated host cells that attack allogeneic targets in the recipient, primarily in the skin, liver and gastrointestinal tract. Analysis of clinical data has shown that leukemia patients who develop GVHD after bone marrow transplantation have a lower probability of relapse compared with patients who do not develop GVHD (2-6). Graft-versus-leukemia (GVL) effect is believed to contribute substantially to the mechanism by which leukemia patients receiving an allogeneic marrow transplant may be cured (7). Unfortunately, this advantage of allogeneic over autologous bone marrow transplantation often is offset by the mortality that results from GVHD in allo-
cell activity against Daudi and K562 targets (18, 19). Primitive hematopoietic cells are initially well maintained under standard conditions of LTC (100% recovery after the first 10 days [17]; therefore, a first series of experiments was undertaken to compare BM-LAK cell activity from seven-day cultures of marrow maintained in this way with marrow cells incubated in medium supplemented only with 25% FCS, $10^{-4}$ M 2-mercaptoethanol and 1000 unit/mL IL-2. Similar activity was generated with either culture medium (Figure 2). This result is noteworthy insofar as LTC medium contains, in addition to 12.5% FCS and $10^{-4}$ M 2-mercaptoethanol, 12.5% horse serum and $10^{-6}$ M hydrocortisone, two constituents that might affect LAK cell activation. Conversely, the presence of IL-2 in the LTC medium did not have any significant effect on the number of LTC-indicating cells recovered from seven-day-old LTC of normal marrow (116±25% of input LTC-initiating cell numbers in cultures plus IL-2 versus 147±21% of input LTC-initiating cell numbers in controls).

A series of subsequent experiments using the same endpoints of BM-LAK cell activity and primitive hematopoietic cell numbers was then undertaken to consider various additional practical or theoretical considerations. The first was to compare starting marrow cell suspensions obtained by density centrifugation on Percoll (Pharmacia, Fine Chemicals, New Jersey) (density 1.070 g/cm$^3$) compared with the light density fraction obtained by standard density centrifugation on Ficoll-Hypaque (density 1.077 g/cm$^3$). This was important to establish in anticipation of using a bulk Percoll-based method for separating the desired cells from entire marrow harvests for clinical applications, which was previously developed (20). Previous studies had shown that LTC-initiating cells have a density less than 1.067 g/cm$^3$ (21). Additional experiments revealed

**Figure 1** Schematic presentation of the prospective clinical trial involving culture of bone marrow for seven days in interleukin (IL)-2. During the culture period the patient undergoes conditioning treatment with high dose cyclophosphamide and busulfan. After seven days in culture, the bone marrow cells are recovered and infused into the patient who will also receive IL-2 subcutaneously (SC) for the first week after marrow infusion. BMT Bone marrow transplantation.

**Figure 2** Comparison of bone marrow-lymphokine-activated killer cell activity exhibited by normal marrow harvested from interleukin (IL)-2-containing (1000 units/mL) seven-day cultures using medium supplemented with 25% fetal calf serum (FCS) and $10^{-4}$ M 2-mercaptoethanol ('standard' medium) compared with medium supplemented with 12.5% FCS, 12.5% horse serum, $10^{-4}$ M 2-mercaptoethanol and $10^{-6}$ M hydrocortisone sodium hemisuccinate ('LTC' medium).

**RESULTS**

Initial studies established that good LAK cell activity could be generated by culturing normal marrow aspirate cells for seven days in medium containing 25% FCS, $10^{-4}$ mercaptoethanol and 1000 units/mL recombinant human IL-2 (Hoffmann-LaRoche, Basel, Switzerland), using standard chromium release assays to define LAK cell activity against Daudi and K562 targets (18, 19). Primitive hematopoietic cells are initially well maintained under standard conditions of LTC (100% recovery after the first 10 days [17]; therefore, a first series of experiments was undertaken to compare BM-LAK cell activity from seven-day cultures of marrow maintained in this way with marrow cells incubated in medium supplemented only with 25% FCS, $10^{-4}$ M 2-mercaptoethanol and 1000 unit/mL IL-2. Similar activity was generated with either culture medium (Figure 2). This result is noteworthy insofar as LTC medium contains, in addition to 12.5% FCS and $10^{-4}$ M 2-mercaptoethanol, 12.5% horse serum and $10^{-6}$ M hydrocortisone, two constituents that might affect LAK cell activation. Conversely, the presence of IL-2 in the LTC medium did not have any significant effect on the number of LTC-indicating cells recovered from seven-day-old LTC of normal marrow (116±25% of input LTC-initiating cell numbers in cultures plus IL-2 versus 147±21% of input LTC-initiating cell numbers in controls).

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equivalent BM-LAK cell generation from cultured marrow initially isolated on Percoll compared with marrow isolated using Ficoll-Hypaque (data not shown). The effect of replacing the medium after the first four days of culture, as well as the effect of adding fresh IL-2 at that time, were investigated in anticipation that either of these manipulations might enhance BM-LAK cell generation further. However, this proved not to be the case (data not shown). Conversely, no killing of Daudi cells was seen by bone marrow cells after seven days of culture without IL-2.

It was anticipated that patients would also receive IL-2 in vivo for seven days after infusion of the culture; therefore, IL-2-activated autograft, BM-LAK cell activity was measured after a further seven days in IL-2-containing cultures. Comparison of the LAK activity exhibited by cultured marrow cells at the end of the first seven days of culture with IL-2 and at the end of a second seven-day period of culture with IL-2 showed that this function was retained under these conditions at undiminished levels on a per cell basis (data not shown).

DISCUSSION

Patients with acute leukemia or lymphoma who receive an autologous bone marrow transplant have a higher relapse rate compared with patients who receive an allogeneic transplantation for the same disease and disease state. Although autologous marrow could be considered contaminated with malignant cells, this is unlikely to be the only explanation for this difference in relapse rates. For example, data from twin transplants where the transplanted marrow is free from leukemia show an incidence of relapse post transplant about the same as recipients of autologous bone marrow transplantation. There also is considerable evidence to suggest that certain immune mechanisms that are activated after allogeneic bone marrow transplantation, but not after autologous or twin transplants, contribute to the elimination of residual leukemia (7,22).

As one approach to decreasing relapse rates in a protocol involving autologous bone marrow transplantation, the authors evaluated a procedure for generating autologous BM-LAK cells within the autograft via a seven-day pre-incubation step. This report outlines a number of parameters considered to develop an optimal method for handling and culturing the cells that also is feasible for future clinical application. The results show that culture conditions previously optimized to preserve LTC-initiating cells, the most primitive hematopoietic cells currently detectable (23), are also optimal for BM-LAK cell generation in the presence of IL-2. Moreover, initial evaluation of bone marrow from patients with acute myelogenous leukemia in first remission has shown these to be capable of generating normal levels of LAK activity in seven-day cultures supplemented with IL-2, thus setting the stage for a clinical trial using this approach.


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