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MONOCLONAL ANTIBODIES FOR DIAGNOSIS AND THERAPY OF LYMPHOPROLIFERATIVE DISEASES*

*Dario Campana MD***, *George Janossy MD****

Prevention of GvHD in allogeneic bone marrow transplantation

A number of diseases have become curable with bone marrow transplantation (BMT). In allogeneic BMT, in spite of HLA matching, T lymphocytes of the donor may recognize the host's tissues as foreign and initiate graft-versus-host disease (GvHD). GvHD occurs in approximately 50 % of HLA-matched allogeneic BMT and contributes to the fatal outcome in approximately 25 % of cases[1,2].

During the last few years, lymphocyte depletion *in vitro* has been established as a valuable alternative to the use of immunosuppressive drugs in the prevention of GvHD. Monoclonal antibodies (McAbs) provide powerful tools for specifically removing unwanted cells from the suspension of BM cells.

a) Methods for T cell depletion

Well documented studies in animals have shown that GvHD is caused by T lymphocytes[3,4]. A great number of different "cocktails" of McAbs have been used to remove these cells. However, what is referred to as "T cell depletion" in the literature, is often achieved with reagents which also react with non-T cells. For instance, CD2 reagents identify a subset of NK cells[5] and Campath-1 reacts with a wide range of cells including NK cells, B lymphocytes and monocytes[6]. Few purging methods appear to be both reliable and simple for routine use in clinical work. McAbs and rabbit complement (C)[7,8] is one of these methods. Rabbit serum is now available from several commercial sources. The serum is usually tested for the lytic capacity in the presence of C' fixing McAbs, and it is also tested for the intrinsic toxicity to myeloid and erythroid progenitors identifiable *in vitro*. Thirty-day rabbit complement is preferred because at this age the animals have minimal xenospecific cytotoxicity. An attractive alternative to rabbit C' is the use of McAbs with human C' fixing capacity. In this case the source of C' is the patient's own serum and has the advantage of avoiding the standardization of rabbit serum. Some rat McAbs have a powerful human C' fixing capacity[6]. Mouse McAbs of IgM class also work with human C', although often perform more efficiently with rabbit C'[8]. The use of magnetic beads directly conjugated to McAbs or to second layer antisera[9,10] and immunotoxins[11,12] represent valuable alternatives to the C' methods.

* From the Department of Immunology, Royal Free Hospital School of Medicine, London, England.
** Associate Professor of Immunology, Royal Free Hospital School of Medicine.
*** Professor of Immunology, Royal Free Hospital School of Medicine.

b) Clinical applications

The observations of the clinical applications of McAbs for GvHD prevention are as follows: First, it has been shown that in man, like in animals, GvHD in HLA-matched allogeneic BMT is caused by T lymphocytes alone[7]. Second, the "absolute" elimination of T cells is not necessary and several groups have shown that a depletion leaving $<10^6$/kg T cells to be injected in the patient is sufficient to prevent severe GvHD in HLA-matched patients[7,13]. The elimination of T lymphocytes with the T cell associated McAbs MBG6 or RFT12 (CD6) and RFT8 (CD8) and rabbit C' has effectively prevented severe GvHD in HLA-matched allogeneic BMT[7]. It is not necessary to remove non-T cells, nor to give immunosuppressive drugs post-transplant. In these patients, the adoptive transfer of humoral B cell immunity to tetanus toxoid and hepatitis B has been demonstrated[14].

Two major disadvantages have however been attributed to lymphocyte removal: increase in leukaemia relapse[15,16] and the rejection of grafts. The reported increase in leukaemia relapse after lymphocyte depletion has been observed in studies where T cells (and also non-T cells such as NK cells) were removed and additional immunosuppressive therapy was given post transplant. Thus, the effect of *specific* T cell depletion compared to that of immunosuppressive drugs (e.g. cyclosporin A) has not yet been evaluated critically.

The second drawback attributed to T cell depletion is an increased incidence of failure to engraft or rejection following engraftment[17]. This phenomenon, however, seems to be controllable by adjustments in the conditioning regimen. A recent report illustrates this point. 7 x 2 Gy fractionated irradiation plus cyclophosphamide have yielded take in 26 out of 27 patients receiving HLA-matched T cell depleted marrow[18]. Other regimens such as 7.5 Gy fast rate single dose and cyclophosphamide also seem to ensure good takes[8]. However, when lower total body irradiation (TBI) doses have been delivered (6.9 - 7.2 Gy single dose or 6×2 Gy fractionated), lack of engraftment and rejection has become a problem in an unacceptably high number of transplant patients[18]. Thus it appears that safe 'takes' and prevention of GvHD are achievable in HLA-matched allogeneic BMT although the therapeutic window remains very narrow. A further increase in TBI is associated with considerable toxicity.

This is of particular relevance in mismatched-haploidentical BMT: here GvHD is more difficult to fully prevent and lack of engraftment is more frequent. Thus additional measures are required, without increasing drug-related toxicity. Alternatives such as temporary anaesthesia of the recipient's immune system with HLFA McAbs have produced encouraging results in immunodeficient patients[19]. However, this approach does not appear to be sufficient for

decreasing the complications of haploidentical BMT in patients with leukaemia or aplastic anaemia. Perhaps additional anti-T cell McAbs could help in this field.

A final observation on the effects of specific T cell depletion comes from those studies in which the regeneration of the immune system has been investigated. A slow recovery of T cells has been observed during the 60-150 days post transplant[13]. In previous studies, when T cell depletion was not performed, the absolute number of CD8 cells in peripheral blood rapidly reached abnormally high levels. This resulted in particularly low T4/T8 ratios[20]. The differences of CD8 reconstitution between T cell depletion and other studies may be related to the different regeneration patterns of T cell precursors as opposed to that of inoculated mature cells[13].

Removal of residual malignant cells in autologous BMT

Autologous BMT is associated with the risk of reinjecting neoplastic cells with the patient's own marrow. The number of neoplastic cells present in a BM in "complete remission" is unknown. The concept of "complete remission" is poorly defined, when one considers that different criteria (immunological, cytogenetic and molecular) may be used for identifying residual disease but none of these parameters has been used systematically in assessing the lack of residual leukaemia in the re-injected BM. In most cases of autologous BMT, there is no morphological evidence of BM involvement, and the need of using additional purging methods other than conventional chemotherapy, is based on theoretical considerations rather than facts.

A proportion (1-2%) of the total BM pool is sufficient to reconstitute haemopoiesis after autologous BMT. By using such a small volume of BM harvested in "remission", a dilution effect of malignant cells takes place. Thus, autologous BMT, even without purging, as a rescue after intensive chemoradiotherapy is a rational form of curative anti-neoplastic therapy.

a) The phenotype of malignant lymphoid cells

Phenotypic studies have an important practical application in the diagnosis and therapy of leukaemia and lymphoma. The extensive study of the reactivity of different McAbs on leukaemia and lymphoma blasts has produced simple diagnostic panels[8,21]. The studies on both normal and neoplastic lymphoid cells support the view that particular attention should be paid to the methodology employed. It is well known that in common acute lymphoblastic leukaemia (cALL) and in T-ALL blasts reflect the phenotypes of normal lymphoid precursors in the BM and the thymus, respectively (reviewed in ref. 22). Thus, with methods which permeabilize the cell membranes, cytoplasmic markers such as CD22 for null-ALL and cALL and CD3 for T-ALL should be preferred. In contrast, early surface

antigens such as CD19 and CD7 are optimal when intact cells are labelled in cell suspension. We have compared the cytoplasmic versus surface expression of CD22 (RFB4) and CD3 (CD3a: UCHT1; CD3c OKT3) on 40 cases of null and common ALL. 29 cases of $sCD3^-$ T-ALL and 17 cases of acute myeloid leukaemia (AML) including TdT^+ and/or $CD7^+$ cases[23]. The results of this study are as follows: Firstly, all cases of AML were negative for both cCD22 and cCD3 confirming the lymphoid association of these molecules. Secondly, 2/40 cases of null and common ALL were surface $CD22^+$ and 36 of the remaining 38 cases were $cCD22^+$. T-ALL blasts on the other hand did not express cCD22. Thus, cCD22 is a reliable marker for early cells of the B lineage confirming independent studies[24]. Finally, 29/29 cases of $sCD3^-$ T-ALL were $cCD3^+$. In one case, only UCHT1 (CD3a) was positive while OKT3 (CD3c) was negative. The same blasts also showed an unusual surface phenotype because they strongly expressed Class II. Thus this case may have represented the earliest form of T-ALL in our series. Blasts in null-ALL and cALL were invariably $cCD3^-$, confirming that these antigens are only present in T-lineage cells.

Certain types of leukaemias, such as T-ALL and terminal deoxynucleotidyl transferase $(TdT)^+$ AML express a phenotype which is not found in normal BM. Combinations of McAbs can therefore be used to identify these cells and monitor the remission status of the patient. However, this detailed analysis cannot be performed in many leukaemias and lymphomas since the blasts show phenotypic features which are also found on normal cells in the BM. Methods such as cytogenetics and gene rearrangement studies lack sufficient sensitivity. For these reasons, an answer to the question of the utility of purging will only come from comparative clinical trials.

b) Methods of assessing cytoreduction in leukaemia and lymphoma

Early studies using cell lines of cALL origin and B cell lymphoma origin show that it is possible to remove blasts from normal BM cells with appropriate McAbs and C'[25,26]. However, leukaemic cell lines are suitable only for testing reagents in pre-clinical studies and differ from leukaemic cells in many aspects.

A few "clonogenic assays" performed with fresh leukaemic cells have however also been reported[27,28]. A common feature of these tests is the detection of cells which rapidly divide during early phases of culture. These tests are often cumbersome and difficult to reproduce, and also depend upon the addition of selected batches of factors and animal sera. It is therefore relevant to standardize reproducible and sensitive methods which allow the assessment of the efficacy of blast removal using fresh leukaemia and lymphoma cells, and also to test whether such removal also eliminates the proliferative fraction of these populations.

We have described a technique which is based on the use of independent markers such as TdT for ALL, and Ig or cD22 for B cell lymphoma. The detection of residual target cells is performed with immunofluorescence methods. Malignant cells are mixed with "inert" cells (e.g. the patient's own red blood cells) in approximately equal numbers. The efficacy of lysis can then be assessed by comparing the number of TdT^+ (or Ig^+, $cCD22^+$) cells in the sample after C' lysis with their number in a control (irrelevant Ab) sample for a given number of "inert" cells[8].

Double colour immunofluorescence on cytospins has been shown to be a sensitive method for precisely identifying residual blasts. Dilution experiments have previously demonstrated that 1 in 10^4 residual TdT cells could be identified with these methods[29]. We have confirmed these studies with dilution experiments. Double colour IF was compared to single colour cell sorter and microscope analysis, as well as to molecular studies for Ig-gene rearrangements studies in order to assess the efficacy of detecting common ALL blasts admixed with normal peripheral blood. The double colour immunofluorescence technique was shown to be the most sensitive method for detecting residual cALL blasts[23].

c) The selection of optimal reagents

The classification of reagents by the Leucocyte Differentiation Antigens Workshops[30,31,32] have facilitated the task of scientists who wish to use McAbs to eliminate unwanted cells. McAbs recognizing the same epitopes but of different class and subclass can be chosen according to the individual aims.

When a C' lysis system is used, it is important to determine the C' fixing capacity of each individual reagent. We have tested the reagents sent to the Third Leucocyte Differentiation Antigens Workshop and classified within the CD10, CD19 and CD20 clusters for their reactivity and ability to fix human and/or rabbit C'. The conclusions of this study are as follows: Firstly, CD10 McAbs reacted with TdT^+ cells and CD19 Abs reacted with TdT^+, pre-B and $sIgM^+$ cells in the BM. Antibodies of the CD20 cluster and unclustered reagents such as RFB7 and Y29/55 were B cell specific but appeared on the cell surface at a slightly later stage of B cell differentiation. Secondly, these Abs reacted with fresh leukaemia and lymphoma cells representing the corresponding staging of B cell differentiation. Finally, the reagents which performed better in terms of rabbit C' fixation were RFAL3 (CD10), SB4 (CD19), B-C1 (CD20), RFB7 and Y29/55 (unclustered; ref. 33). RFAL3, B-C1 and RFB7 are also human C' fixing while SB4 has little human C' fixing capacity on its own but can improve that of other reagents when used in combination[34]. The conclusion of these studies is that in common ALL and null ALL a combination of RFAL3 and SB4 is optimal, while for the elimination of lymphoma cells a combination of RFB7 and SB4 with the possible addition of

other reagents such as B-C1 or Y29/55 can be recommended. Independent studies have also shown the suitability of the CD19-CD20 cocktail for B cell lymphoma[35]. Most of these detailed investigations have concentrated on the comparison of C' fixing McAbs.

d) Investigation of individual patients

The antigenic expression of malignant cells is heterogeneous. Cocktails of Abs are used in order to overcome this heterogeneity. We have investigated the elimination of leukaemia and lymphoma cells with the different combinations of the McAbs selected and human and/or rabbit C'. These investigations are performed on fresh leukaemia and lymphoma samples. When lymph nodes of lymphoma patients are studied, a cell suspension is obtained either by treating the nodes mechanically or enzimatically. With these methods we have now studied >100 cases of ALL and B cell lymphoma at presentation or in relapse. The selected McAbs are very efficient in eliminating malignant cells: e.g. with a combination of RFAL3+SB4 and rabbit C' >4 log lysis has been achieved in >70% of null and cALL cases. These Abs also eliminated the proliferative fraction of leukaemic cells (Janossy G, Campana D et al, manuscript in preparation).

e) Clinical observations

Many factors such as the use of more aggressive conditioning regimes and the improvement of cryopreservation and transfusion technology contribute to the success of an autologous BMT. The toxicity of this procedure is now comparable to that of conventional chemotherapy. As a consequence, a number of patients may benefit from an expanding autologous BMT programme.

A series of pilot studies have provided the necessary preliminary information on the effects of purging in autologous BMT. Early clinical studies of purging in ALL and B cell lymphoma using McAbs and rabbit C' [33,36-38] indicated the feasibility and the safety of the procedure. Recently published results in patients with non-Hodgkin's lymphoma with poor prognosis treated with autologous BMT that had been purged with B1 McAb confirm the safety of the procedure in older patients. The median age in this study was 42 yrs[39].

A clinical collaboration has been established between our Department and the groups at the University Hospital, Uppsala and at the Royal Infirmary and Hospital for Sick Children, Glasgow. In this trial the selection of patients is based on the following criteria: (a) ALL patients are transplanted in first remission when at high risk of relapse (e.g. $>100 \times 10^9$ WBC at presentation) or in second or subsequent full remissions. (b) The phenotype of blasts cells is characterized at presentation or in relapse. This is performed with the same reagents which are used for purging, and the sensitivity of the blasts to C' lysis is also assessed using the methods described. The optimal combination of reagents which are found to be

capable of achieving >4 log cytoreduction is used for purging. (c) Additional pieces of information are the detection of residual target cells after purging with sensitive methods and, (d) the assessment of the CFU-GM injected into the patient. Into this pilot trial 19 patients (11 children and 8 adults) have been entered in first (4 patients) and second (14 patients) or subsequent (1 patient) remissions. The follow-up is >30 months. Sixteen out of 19 patients are alive and well. Thirteen of these are now beyond 12 months follow-up. In 4 other patients conditioning prior autologous BMT was carried out in relapse. All 4 patients in this second group relapsed within the first year after BMT.

A number of conclusions can be drawn from this study. Firstly, post transplant complications were minimal confirming the low toxicity of the procedure. Secondly, the purging procedure effectively eliminated target cells even in patients with detectable BM relapse but still allowed a hemopoietic recovery. This recovery was as fast as seen in patients who received unpurged BM[40]. Finally, no early relapses have been observed in the patients transplanted in remission. The 3 relapses occurred after 9, 10 and 12 months of transplant. We tentatively conclude that many early relapses seen in other studies might be due to considerable residual disease which may be detectable at the time of transplant with the immunological techniques used in our study.

At this stage, it will be necessary to organize large collaborative trials in order to directly compare autologous BMT protocols with or without purging. To obtain a credible answer from these studies, the purging methods employed must be of proven efficacy and safety.

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