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Gene Marking After Bone Marrow Transplantation

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INTRODUCTION

BONE MARROW stem cells are desirable targets for gene therapy, since their genetic modification could treat many inherited and acquired diseases. Marrow progenitor cells are also appealing targets for logistic reasons; the cells are easily obtained and handled *ex vivo* and readily returned. Most importantly, genetic modification of just one single stem cell could, in principle, be sufficient to repopulate the haemopoietic and lymphoid system of an individual for their entire life. However, most pre-clinical experiments have suggested that currently available methods for transferring, expressing and regulating new genetic material have too limited an efficiency for clinical use [1, 2]. The problem has arisen because exploitation of the advantages of marrow stem cells for gene therapy requires the use of a vector which will stably integrate in the host cell genome and thereby retain its presence, not only in the original stem cell, but in the multitudinous progeny thereof. At present, retroviruses are the only vectors capable of achieving such integration, but will behave in this way only in dividing cells. Because the great majority of marrow stem cells at any one time are non-cycling, transfer efficiencies have been exceedingly low, and the expression levels obtained would have been wholly inadequate to obtain any clinical benefit. Because gene transfer carries a finite risk to the patient, there can be no justification for opening a protocol in which the risk:benefit ratio was infinitely high [1, 2].

These limitations led us to propose a series of gene marking experiments, using recipients of autologous bone marrow transplantation (ABMT) [3, 4]. In these patients, clinically relevant issues could be addressed, even with the extremely limited efficiency of gene transfer then available. Our initial aim was to use gene marking techniques to discover the source of relapse after ABMT, and to learn more about the biology of normal marrow reconstitution. We have also begun to use gene marking in patients following allogeneic bone marrow transplantation as part of a study aimed at adoptively transferring specific T cell immunity against Epstein–Barr virus (EBV)-infected B-cells, in

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an attempt to prevent the fatal, EBV-driven, lymphoproliferative disease which may occur in up to 30% of the recipients of these transplants.

Source of relapse after autologous bone marrow transplantation

While the dose intensification permitted by autologous bone marrow transplantation appears to result in responses in a number of malignant diseases, including myeloid neoplasia [5–7], disease recurrence remains the major cause of treatment failure. The possibility that the harvested marrow may contain residual malignant cells has led to extensive evaluation of techniques for purging marrow prior to storage and subsequent reinfusion [8–12]. Animal and preclinical human studies have shown that these methods do reduce contamination with malignant cells that have been deliberately added to marrow, but no randomised clinical trials have shown a significant reduction in the risk of relapse in patients receiving purged marrow [12–16]. This is a significant issue as all purging techniques currently available are non-selective and also damage normal progenitors. The consequent delay in haemopoietic recovery results in increased morbidity and mortality.

One approach to resolving this issue is to transfer marker genes into residual malignant cells in the marrow prior to reinfusion. If gene-marked malignant cells subsequently became detectable in the marrow or peripheral blood in patients who relapsed following autologous transplant, this would be direct evidence that the harvested marrow contributes to disease recurrence. Moreover, the finding of marked cells at relapse would permit subsequent evaluation of *ex vivo* purging techniques for their ability to eradicate clonogenic cells [3, 4]. Thus, for the first time it would be possible to directly compare the efficacy of the numerous available techniques for marrow purging.

Feasibility of gene marking

Gene marking of clonogenic malignant cells in bone marrow can readily be demonstrated *ex vivo*. Recent studies of acute and chronic myeloid leukaemia (AML, CML), acute lymphoblastic leukaemia (ALL) and neuroblastoma have shown that clonogenic cells can be marked with an efficiency of 0.1–40% [3, 4]. In the initial clinical marking studies, safety concerns demanded that only an aliquot of the marrow intended for re-infusion was transduced, so that between 1 and 10% of any putative malignant cells in the marrow would usually be marked by this approach. The obvious question is whether the marker gene could ever be detected at subsequent relapse, given this low efficiency of marking clonogenic cells. The answer depends on how many malignant cells in the "remission" marrow contributed to the relapse. If fewer than 10 cells were involved, the chances of detecting a "marked relapse" are rather small. With larger numbers of cells contributing, the probability of detection improves substantially. Results of minimal residual disease (MRD) studies indicate that many "remission" marrows will, in fact, contain appreciably more than this minimum number of malignant cells [17]. Detection of MRD is most sensitive in ALL and CML, where unique gene rearrangements allow PCR techniques to detect a single residual malignant cell in amongst 100 000 normal cells. Since a 70-kg patient will receive a minimum of 7×10^9 marrow cells at ABMT, a reinfused "remission" marrow may in fact contain seventy thousand residual malignant cells. For AML, MRD detection techniques are orders of magnitude less sensitive, and thus hundreds of thousands or even millions of blasts may be present in "remission" marrow.

Growth of normal progenitor cells in transplanted marrow—implications for gene therapy

The gene transfer techniques used to mark malignant clonogenic cells will also mark long-lived normal marrow precursor cells. Thus, the technique was expected to allow the growth of autologous transplanted marrow to be followed and for repopulation with infused marrow to be distinguished from the recovery of surviving progenitors in the host. It would then be possible to use marked cells to determine the influence of haemopoietic growth factors on the recovery of autologous marrow, and to develop effective methods of treating autologous marrow before transplant, to increase the speed with which reconstitution occurs. Similarly, it should be possible to determine which growth factor combinations are able to increase the efficiency of transduction of normal progenitor cells. This information will be essential to the success of therapeutic gene transfer protocols.

OUTLINE OF CURRENT STUDIES

All gene transfer studies were approved by the institutional review board and biosafety committee of St Jude Children's Research Hospital and the recombinant DNA advisory committee of the National Institutes of Health. Written informed consent was given by the patients or their parents. Gene marker studies using bone marrow of patients with AML and neuroblastoma began in September 1991 and January 1992, respectively.

We studied 21 patients, 2–19 years of age, who were candidates for ABMT AML ($n = 12$) or neuroblastoma ($n = 9$). Remission induction therapy has been described previously [18]. Nucleated bone marrow ($> 1.5 \times 10^8$ cells/kg of body weight) was taken from the posterior iliac crest and two thirds were cryopreserved immediately. The remaining third was separated on a Ficoll gradient to produce a mononuclear cell fraction. Either the LNL6 or the closely-related G1N retroviral vector, both of which contain the Neo^R gene, were added to marrow cells in culture bags (SteriCell, DuPont) as described previously [3]. Haemopoietic growth factors were not added. After 6 h of incubation at 37°C, the bone marrow cells were retrieved, washed and cryopreserved after removal of an aliquot for microbiological analysis and for assessment of transduction efficiency. Prior to transplantation, AML patients were conditioned with busulphan (16 mg/kg) and cyclophosphamide (50 mg/kg × 4), while neuroblastoma patients received carboplatinum (700 mg/m^2 × 3) and etoposide (500 mg/m^2 × 3). At the time of transplantation, both transduced and unmanipulated marrow cells were thawed and re-infused through each patient's central venous line.

To assess the efficiency of gene transfer and expression in marrow progenitor cells, both pre- and post-transplantation, we obtained mononuclear cells from peripheral blood or bone marrow, separated them on Ficoll–Hypaque gradients and cultured them in methylcellulose as described previously [3]. Transduced cells were cultured with or without the G418 neomycin analogoue at 1 mg (active)/ml, a concentration established as inhibitory to the growth of non-transduced patient progenitor cells and malignant blasts [3]. Colonies, defined as collections of > 50 cells, were identified and counted at 12–14 days on duplicate or triplicate plates, with the results averaged. They were classified by standard techniques as granulocyte–erythroid–monocyte/macrophage (GEMM), granulocyte–monocyte (GM), granulocyte (G), monocyte (M), burst-forming unit-erythroid (BFU-E) or colony-forming unit-erythroid

(CFU-E); immunological or histochemical staining was used to confirm results for selected colonies.

Amplification with the polymerase chain reaction (PCR) was used to detect the transferred Neo^R gene in individual colonies or in bulk populations as described previously [3]. The PCR-amplified products were analysed by gel electrophoresis (1.5% agarose) and Southern blotting with a ^{32}P-labelled Neo-specific probe. Semi-quantitative PCR was also used, with 25 cycles of amplification corresponding to linear signal strength. In some studies, PCR was performed on cDNA using a commerically available kit (Cetus) with identical primers and amplification conditions.

Origin of relapse

In the AML study, 3 of 12 patients have relapsed. In 2, the malignant cells contained the marker gene. Patient 1 relapsed with blasts in the peripheral blood at day 80 post-transplant. Her blasts co-expressed CD34 and CD13 and the Neo^R gene was detected in both bulk sorted populations and in G418-resistant colonies formed when these cells were plated in clonogenic assays. Patient 2 was found to be in relapse on a routine bone marrow at 6 months. This patient had two leukaemia-specific markers. First, her blasts co-expressed CD34 and CD56, a combination not found on normal haemopoietic cells [19].

Second, she had a complex t(1:8:21) translocation resulting in generation of an AML1/ETO fusion transcript that could be identified by PCR. We were, therefore, able to sort blasts expressing CD34 and CD56 and show co-expression in a single clonogenic cell of both a leukaemia-specific marker (the AML:ETO fusion protein) and the transferred neomycin gene (Figure 1) [18].

Similarly, three neuroblastoma patients have relapsed, all with gene marked neuroblastoma cells. In these patients, identification of marked neuroblastoma cells was confirmed by detection of co-expression of neuroblastoma-specific antigens (for example, GD2) together with the transferred marker gene. Two of the neuroblastoma patients relapsed in their marrow, but the third had disease recurrence in a new site, the right lobe of the liver. Biopsy of this extra-medullary site showed the presence of gene marked neuroblasts (Figure 2).

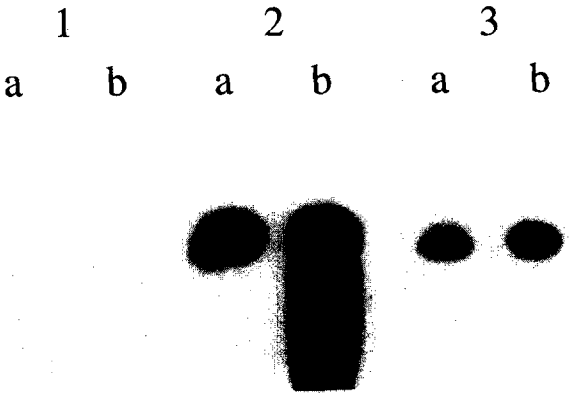

Figure 1. Southern blot of polymerase chain reaction analysis of cDNA from CD34+ CD56+ 1:8:21-bearing cells and blast colonies derived from that population. Lane 1a/1b represents negative control (105 remission marrow cells pretransduction); lane 2a, Neo^R in 10^5 CD34+ CD56+ cells; lane 2b, the 8:21 fusion product from the same CD34+ CD56+ cells; lane 3a, Neo^R in a single colony grown from CD34+ CD56+ cells, and lane 3b, the 8;21 fusion product from the same colony.

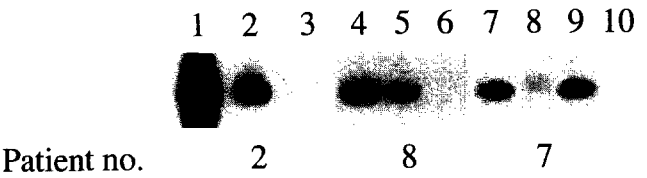

Figure 2. PCR analysis of GD2+CD45− neuroblastoma cells and colonies at the time of relapse. PCR amplification was performed on DNA from 10^4 sorted GD2+CD45− cells from the site of relapse and from individual, G418-resistant colonies grown in methylcellulose (see text). Patient 2: lane 1 = marrow tumour, lane 2 = G418-resistant colony, lane 3 = negative control. Patient 8: lane 4 = marrow tumour, lane 5 = G418-resistant colony, lane 6 = negative control. Patient 7: lane 7 = liver tumour, lanes 8,9 = G418-resistant colonies (liver, marrow), lane 10 = negative control. The band shown is the 720-bp meomycin resistance gene amplification product.

These data show definitively that marrow harvested in apparent clinical remission of both haematological and non-haematological malignancies may contain residual tumorigenic cells and that these cells can contribute to disease recurrence at both medullary and extra-medullary sites. Reduction in relapse risk will therefore require, *inter alia*, the development of effective purging techniques. Gene marking may help in this aim. We have begun our studies of marrow purging, using two gene markers to compare marrow purging versus no purging, or two different purging techniques. We are using two closely-related vectors, G1N and LNL6, which can be discriminated by virtue of differing fragment sizes they produce after PCR amplification. In the AML study, one third of the marrow is frozen unpurged as a safety backup. The remaining marrow is split into two aliquots which are marked with G1Na or LNL6 and then randomly assigned to purging with 4HC or IL2. At the time of transplant, both aliquots are re-infused. If the patient should subsequently relapse, detection of either marker will allow us to learn if either of these purging techniques is effective (Figure 3). 3 patients have been treated on this protocol as of December 1993, but no data are yet available. A similar protocol for neuroblastoma will begin accrual imminently [20].

Clearly, however, the full exploitation of the approach will require development of highly efficient means of transferring marker genes to malignant cells, so that it will be possible to

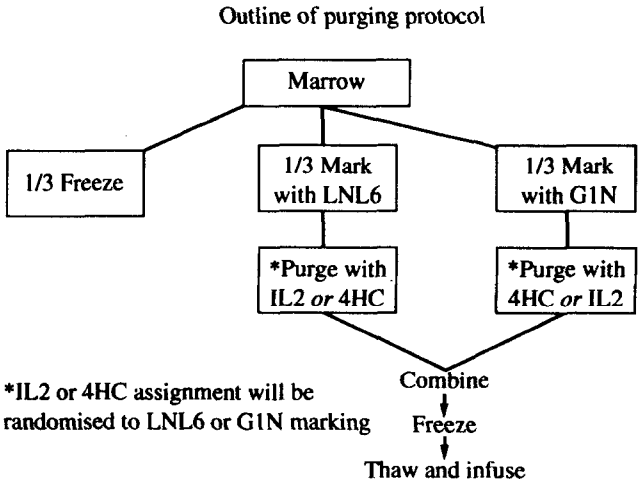

Figure 3. Scheme for double marker analysis of the efficacy of purging in AML.

detect a contribution from residual tumorigenic cells in marrow even if the numbers remaining after purging are very small.

Gene transfer to normal cells

2 of the 21 patients died (of disease progression and sepsis) within 21 days of transplantation and could not be further assessed. In the remaining 19 patients, there were no indications that laboratory manipulation of marrow specimens had adversely affected engraftment. The median times to recovery of 500 neutrophils/ml were 36 (AML) and 31 days (neuroblastoma); the equivalent times to reach 2×10^4 platelets/ml (unsupported) were 36 and 30 days. None of the patients has shown evidence of malignant transformation or other complications that could be attributed to the gene transfer process [21].

In 15/19 patients, 1 month after ABMT, the presence of the gene in haemopoietic progenitor cells *in vivo* was confirmed, using clonogenic assays, that showed gene-marked progenitor cells. The contribution of infused marrow to long-term haemopoietic recovery was shown in patients with follow up of 6 months or more after transplantation. G418-resistant progenitor cells persisted for 6 months in 8/9 patients and for 1 year in 5/5; marked progenitor cells remained detectable in marrow from 2 patients (1 AML and 1 neuroblastoma) who have survived for more than 18 months post-transplantation (Figure 4). In 3/9 patients, multi-lineage GEMM colonies became apparent after transplantation (Figure 4) [21]. The presence of the marker gene in these individual G418-resistant colonies was confirmed at all time points by PCR analysis.

The presence and long-term expression of the marker gene also occurred in the mature progeny of marrow precursor cells, including peripheral blood T and B cells and neutrophils and was detected in lymphoblastoid cell lines established from 6/8 patients and in cytotoxic T cell lines in 8/9 patients. There was variation in the level of transfer (the percentage of G418-resistant myeloid colonies ranging from 2 to 14%) and the expression between the different lineages, it being higher in myeloid cells than in T lymphocytes and lowest in B lymphocytes. These levels of transfer are higher than predicted from animal models and may be attributed to the fact that marrow was harvested during regeneration after intensive chemotherapy, when a higher than normal proportion of stem cells are in cycle.

These data also support a substantial contribution to marrow reconstitution from autologous transplants, and suggest that this contribution includes long-lived multipotent stem cells. It should be possible to use the genetic marker technique we describe to compare the regenerative potential of marrow versus peripheral blood-derived progenitor cells, and hence discover their relative value for haemopoietic rescue. Genetic marking could also be used to determine directly which *ex vivo* or *in vivo* combination of cytokines will increase the entry of long-term marrow repopulating cells into cell cycle and thereby reduce the period of marrow hypoplasia and immunodeficiency that follows autologous stem cell transplantation.

Gene marking of adoptively transferred EBV-specific CTLs

We have also begun a marking study in recipients of allogeneic bone marrow obtained from matched unrelated or mismatched family members donors. In these individuals, there is greater

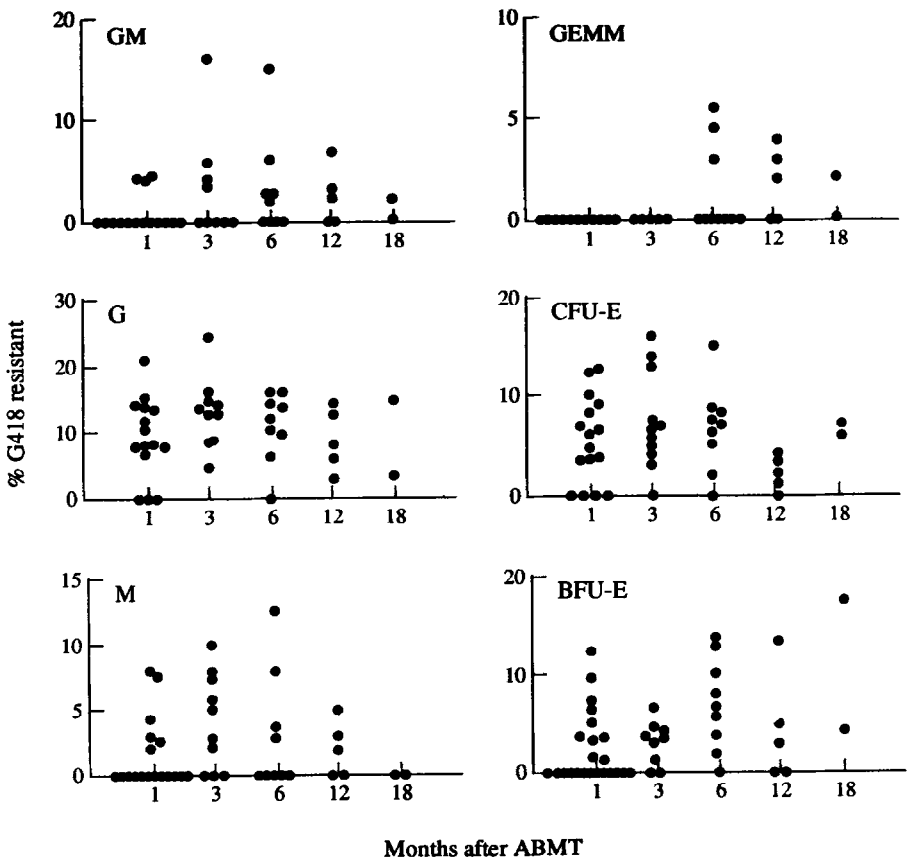

Figure 4. G418-resistant colonies as a percentage of the total colonies in transduced marrow at various time points after transplantation. The procedures for establishing cultures and determining the extent of colony formation in the presence of the G418 analogue are described in the text, as are the abbreviations of cell types.

genetic disparity between donor and recipient and a higher risk of GVHD. *In vitro* T-cell depletion of donor marrow is an effective means of reducing the risk of BMT, but recipients of depleted marrow have delayed immune recovery and an increased incidence of viral infections. One of the most lethal of these is Epstein–Barr virus-driven lymphoproliferative disease (EBV-LPD). EBV usually persists lifelong after primary infection, by a combination of chronic replication in the mucosa and latency in peripheral blood B cells; this carriage is generally asymptomatic. However, if cellular immune responses are impaired, for example, after T-cell-depleted allogeneic BMT, EBV-infected B-cells may undergo unresticted proliferation and produce lymphoproliferative disease. This complication occurs in 5–30% of patients receiving marrow from mismatched family members or unrelated donors and is almost invariably lethal [22].

Since EBV-LPD only develops because of the absence of anti-viral T-cell effector function, the therapeutic benefits of re-infusing donor T-cells has recently been explored. We, and others, have found that such therapy can induce disease regression, although with a high risk of inducing significant graft versus host disease (Figure 5). To avoid this latter complication, we are currently evaluating infusions of EBV-specific donor T-cells in high-risk patients. These specific T-cells are generated by culturing donor T-cells with donor-derived EBV-infected lymphoblastoid cell lines. To learn more about the fate, distribution and anti-viral activity of the EBV-specific CTL, we are marking them with the neomycin resistance gene before administration. At the time of writing, 8 patients have been treated and the results are being evaluated. It may be possible to use a similar approach to transfer T-cells specific for other malignancies with tumour restricted antigens.

CONCLUSION

Genetic marking can be used to evaluate the contribution of residual tumorigenic cells in marrow to relapse after autologous bone marrow transplantation. Marker studies should also prove valuable for assessing the efficacy of purging and for learning about the impact of haemopoietic growth factor treatment of harvested marrow or peripheral blood stem cells on subsequent progenitor cell growth and development *in vivo*. Finally, gene marking should help evaluate the toxicity and efficacy of T-cell adoptive transfer in recipients of allogeneic BMT.

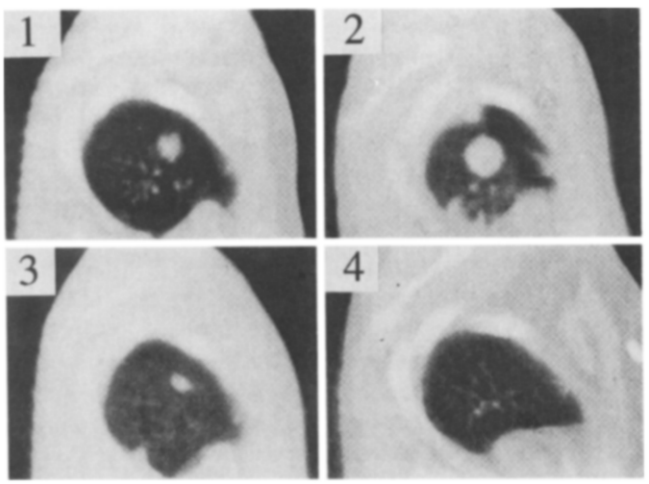

Figure 5. Computed tomography (CT) scan of chest of post-transplant patient; (1) at the time of diagnosis of EBV-LPD; (2) 2 weeks later at the time of infusion of 10^6 donor T cells/kg body weight; (3) 6 weeks after infusion. (4) Subsequent CT scans were reported as normal.

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Cancer Escape from Immune Surveillance: How Can it be Overcome by Gene Transfer?

C. Bruyns, C. Gérard and T. Velu

INTRODUCTION

IT IS generally accepted that the spontaneous generation of cancer cells is a common event, and that the immune system assures a strict surveillance: the detection of a mutant cell leads to its rapid elimination by immune mechanisms, and prevents its progression to clinically detectable disease. A tumour thus develops only when a mutant cell escapes the immune surveillance [1, 2]. T lymphocytes are critical in controlling such anti-tumour immune responses. Recently, a better understanding of the molecular events of antigen presentation and lymphocyte activation in animal models has led to the identification of epitopes on tumour cells that can be recognised by T-cells. It now appears unequivocal that spontaneous human tumours also express tumour-specific antigens that are recognised by antibodies and/or by T-cells. Many of these are encoded by normal cellular genes and are recognised because of over-expression or aberrant expression [3]. Tumour antigens may also be encoded either by mutated cellular genes, or by viral genes. Such antigens may be weak rejection antigens failing to induce effective T-cell responses, either because they share a high homology with self epitope, or because the local environment surrounding the tumour cell may fail to support an immune response or be immunosuppressive. Tumour cells can, in fact, escape or fail to elicit tumour-specific immune responses by various mechanisms.

HOST T-CELL IMPAIRMENT

Even when cancer cells express an antigenic molecule, the host may not respond to the tumour because of a selective deletion or suppression of some T-cell populations. For instance, selective deletion of mature peripheral Vβ2+ T-cells (mostly CD4+) was observed in Balb/C mice inoculated with preneoplastic and neoplastic mammary carcinomas [4]. This phenomenon, similar to that reported for superantigens of either bacterial or viral origin, was shown by adoptive transfer experiments, to be mediated by host cells and to be related to the presence of a mouse mammary tumour virus (MMTV). Likewise, a phenomenon of T-cell unresponsiveness or anergy in a human tumour microenvironment exists, but has not yet been extensively investigated. It is well established that many human tumours are infiltrated by T-cells that can express activation antigens, such as major histocompatibility complex (MHC) class II antigens (HLA-DR) and interleukin (IL)-2 receptors [5]. Surprisingly, however, when fresh tumour-infiltrating lymphocytes (TIL) are isolated from human solid tumours and tested *in vitro* for antitumour function, they show poor cytotoxicity against autologous tumour or other tumoral targets, and fail to proliferate in response to T-cell mitogens. This anergic state seems to vary between tumours, and is reversible upon isolation of TIL and culture in the presence of exogenous IL-2 [6–8]. Therefore, although this possibility cannot be definitively ruled out, it seems unlikely that the lack of immune competence of TIL results from a selective deletion of certain subsets of tumour antigen-reactive T-cells. Rather, the T-cell unresponsiveness would mainly be due to a tumour-induced suppression, operating perhaps through a downregulatory network of T-cells and cytokines.

The requirement of both CD4+ and CD8+ T-cells for the induction of immune control on neoplastic growth was demonstrated by the work of Flamand and colleagues [9]. Using selective depletions of lymphocyte subsets *in vivo*, they showed that both CD3+CD4+ and CD3+CD8+ T cells are involved in immune responses to the class I-positive, class II-negative mouse mastocytoma P815. This work highlighted the central role played by CD4+ T-lymphocytes in immune surveillance [10, 11]. Since these cells are activated by exogenous proteins presented in association with class II histocompatibility antigens, sensitisation protocols based on the use of purified tumour antigens may prove useful for increasing the immune response against tumour. This strategy is illustrated by the work of Kündig and colleagues [12], who investigated the induction of immunity to an artificial tumour-associated antigen, the

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