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REVIEW

Biological and immunological aspects of malignant mesothelioma

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Biological and immunological aspects of malignant mesothelioma. M.J. Garlepp, C.C. Leong. ©ERS Journals Ltd 1995.

ABSTRACT: Malignant mesothelioma (MM) is an aggressive tumour, which is strongly associated with previous asbestos exposure and is resistant to all conventional anticancer therapies. An understanding of the biological properties of MM may provide insights into useful therapeutic strategies, and MM cell lines and animal models have been major contributors to our current knowledge of this tumour.

Although karyotypic abnormalities are frequent, there is no clear evidence of a mesothelioma-specific chromosomal aberration. Similarly, there is no evidence of activation or over-expression of a known oncogene, or of the inactivation of currently identified tumour suppressor genes. A number of growth factors, including platelet derived growth factors A and B (PDGF-A and -B), insulin-like growth factor I and transforming growth factor-beta (TGF-β), and some of their receptors, have been reported to be expressed by MM cells, and each has the potential to play a role as a growth stimulant for MM or to modify immune responses to the tumour. Some data support an autocrine role for PDGF-A.

MM cell lines are susceptible to lysis by a variety of immune effector cells, and their growth can often be inhibited by cytokines. The possibility of stimulating an immune response to MM by genetic manipulation of the tumour cells has been investigated using a murine model. The data so far suggest that transfection of allogeneic class I major histocompatibility complex genes or syngeneic class II genes alone is unlikely to induce protective immunity. Expression of the co-stimulatory molecule B7-1 stimulated tumour-specific cytotoxic T-lymphocytes (CTL), and generation of these CTL was associated with delayed tumour development or tumour rejection. Furthermore, some, but not all, B7-1 expressing clones from one murine cell line were able to generate an antitumour response, which conferred resistance to the parental tumour. However, these experiments also illustrated the heterogeneity of immunogenicity which exists both within and between MM tumours. It is likely that genetic modification using the genes for more than one immunologically relevant molecule will be necessary for successful induction of immunity to the least immunogenic examples of this tumour.

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The incidence of malignant mesothelioma (MM) has been steadily increasing over the last 20 yrs [1]. This increased incidence is expected to continue until at least the year 2020, and relates to the widespread mining and use of amphibole asbestos in the recent past and the long latent period (30–40 yrs) for tumour development. The strong association between MM and occupational exposure to asbestos fibres, particularly of the amphibole type, was clearly established in 1960, and has since been repeatedly confirmed [2]. A significant proportion of patients do, however, present without any known occupational exposure to asbestos. The tumour most commonly affects the mesothelium of the pleural cavity, but can also occur in the peritoneal cavity. Once diagnosed, it is invariably fatal and the median time from diagnosis to death is approximately 9 months. MM is resistant to radiotherapy and chemotherapy, and surgery has not, in general proved useful [3].

The development of MM, and of most other tumours, presumably involves a number of steps [4]. The initial events occur at the level of the genome, where it is assumed that mutations or rearrangements occur which predispose to cancer development. These genetic alterations can lead to activation or mutation of oncogenes, or the inactivation of tumour suppressor genes. Progression of the transformed cells to malignancy then ensues, concomitant with evasion of host defences. Knowledge of the biological processes which promote and maintain the uncontrolled proliferation of tumour cells, and the mechanisms which they employ to evade the immune system of the host, may provide a focus for tumour-specific therapeutic regimens.

Genetic alterations in malignant mesothelioma

Cytogenetic analysis of human mesothelioma cell lines, pleural exudate cells or biopsy material has revealed karyotypic abnormalities in the vast majority of cases [5–7]. *In vitro* studies suggest that mesothelial cells are particularly susceptible to chromosomal damage after asbestos exposure [8]. The chromosomal changes may be initiated by the asbestos particles themselves, either as a result of the action of free radicals released after uptake of asbestos fibres by macrophages or mesothelial cells [9], or by direct interaction of the fibres with the chromosomal deoxyribonucleic acid (DNA), chromosomal proteins or the spindle apparatus [10]. Extensive chromosomal analysis has been undertaken to identify regions of the genome which are consistently altered in MM, and which might provide clues to the genetic modifications involved in MM development. Numerical and structural chromosomal aberrations are complex, and there is great heterogeneity both within a tumour from a single patient and between patients [5, 6]. However, analysis of many samples by a number of groups (reviewed in [7]) has provided data which suggest that certain chromosomes may be consistently affected, and some *in vitro* experimental data suggest that the effects of asbestos on chromosomal DNA may not be entirely random [11, 12]. One commonly reported abnormality is polysomy of chromosome 7 [5, 7]. Since the genes for the epidermal growth factor receptor, a proto-oncogene, and for platelet-derived growth factor A (PDGF-A) [13] are encoded on this chromosome, this observation is of potential interest (see below). In one study, the number of copies of the short arm of chromosome 7 was negatively associated with patient survival [5]. Deletions or monosomies involving chromosomes 1 and 3 have also been frequently reported [5–7], and these are of interest because a gene concerned with cellular senescence has been described on chromosome 1 and a tumour suppressor gene involved in several human cancers is believed to be located on chromosome 3 [7]. The recent localization of the DNA mismatch repair enzyme hMLH1 to chromosome 3p21, and the demonstration that mutations of this gene are linked to hereditary cancer of the colon [14], should generate further interest in this region in MM.

Oncogenes

In many tumour types, there is evidence of activation of characteristic oncogenes, and such activation is believed to be a key element in tumour development and progression [4]. One approach to the treatment of cancer would be to target these genes, or their products, using antisense technology or immunotherapy. Despite intensive investigation there is no published evidence of mutations of the known oncogenes, either in human MM or in asbestos-induced mesotheliomata in rodents. Some evidence of activation of an unknown non-*ras* oncogene has been reported [7], and deregulated expression of *c-myc* was described in murine MM cell lines, although there was no evidence of amplification of the *c-myc* gene in these cell lines [15], or in human mesothelioma cell lines (Christmas and Garlepp, unpublished).

Tumour suppressor genes

An alternative therapeutic strategy, which has been proposed, is the restoration of absent or dysfunctional tumour suppressor gene activity by gene transfer. At present, there is no evidence for consistent mesothelioma-specific aberrations of known tumour suppressor genes. Although the majority of murine asbestos-induced mesotheliomata have been reported to display aberrations of the p53 gene [16], and some reports have described readily detectable p53 protein in human MM tumour samples [17, 18], only a minority of human cell lines have demonstrable mutations or deletions of this gene [19, 20]. The retinoblastoma gene appeared to be expressed normally in human cell lines [21], and although the Wilm's tumour gene, WT-1, [22] was expressed in the majority of human and rodent mesothelioma cell lines ([23] and Versnel, personal communication) genomic analysis of DNA from many samples has revealed a mutation in only one case, an unusual peritoneal mesothelioma which was probably not asbestos associated [24]. Thus, the analyses of oncogenes and tumour suppressor genes carried out to date have not defined potential targets for MM-specific therapy.

Growth factors

Expression of a variety of growth factors and their receptors has been demonstrated both in human and murine mesothelioma cell lines (reviewed in [7, 25]), and each has the potential to act in an autocrine manner. PDGF-A and -B have been subjected to the most study, and some evidence has accumulated to implicate either or both in an autocrine pathway. Transcripts for both PDGF chains are readily demonstrable [26–29], and PDGF-like activity has been demonstrated in the supernatants of cultured cell lines [27, 30]. Furthermore, the transfection of the gene for PDGF-A into a transformed but nonmalignant mesothelial cell line was sufficient to make this line tumorigenic [30]. *In vitro* experiments, in which antisense oligonucleotides to PDGF-A inhibited the proliferation of MM cell lines, have provided support for a role for PDGF-A in MM proliferation [28, 29]. A role for PDGF-B has been proposed by others [31, 32]. Expression of the PDGF receptor-β (PDGFR-β) is upregulated in MM cell lines compared to normal mesothelial cells, and the reverse occurs for PDGFR-α [32], although messenger ribonucleic acid (mRNA) for PDGFR-α is detectable in these cell lines [28]. *In vivo*, PDGFR–α may be upregulated on MM cells by basic fibroblast growth factor [33], which can be expressed by MM cells [34], and may also be released by other cells in the tumour environment.

A second growth factor produced by the majority of MM cells (human and rodent), which may influence

tumour growth and survival in a number of ways, is transforming growth factor-β (TGF-β) [35]. This cytokine exists as multiple isoforms and expression both of TGF-β1 and TGF-β2 has been demonstrated in MM cell lines [27, 36]. The transfection and expression of antisense constructs in a murine MM cell line have provided some data suggesting that inhibition of TGF-β2 can alter MM growth *in vivo*, and that inhibition of either TGF-β1 or TGF-β2 expression can alter the *in vitro* growth characteristics of this cell line [36]. Inhibition of TGF-β expression was, however, only partial in these experiments. Whether complete inhibition of production of this growth factor would produce a more marked effect remains to be determined.

These data suggest that antisense nucleic acids have the potential to influence tumour growth both *in vivo* and *in vitro*. There are, however, several difficulties associated with the *in vivo* application of antisense or tumour suppressor gene constructs. These include the necessity to deliver the construct to every tumour cell, difficulties in ensuring the expression of sufficient antisense transcripts to provide adequate inhibition of growth factor production, and the targeting of these constructs.

Genetic manipulation to enhance antitumour immunity

An ideal approach to the treatment of MM would be to enlist the host immune system to eradicate tumour cells. A great deal of evidence has accumulated over many years to suggest that the immune system can play a role in tumour rejection, and that T-lymphocytes are the major players in such responses [37, 38]. These data have been derived both from human and experimental systems, and include such observations as the infiltration of tumours by T-lymphocytes, the spontaneous remission of tumours, such as neuroblastomas, and the regression of metastases after removal, or successful treatment, of the primary tumour. Furthermore, several groups have now been able to derive tumour-reactive T-cell clones both from human and murine tumours, and such clones have been used to adoptively transfer tumour immunity in experimental animals [37, 39]. More recently, several tumour antigens have been identified, and these have been shown to be recognized by T-cells derived from tumour bearing patients [40]. Despite the evidence that it is possible to mount an immune response to a tumour, why do tumours develop and progress? In order to address this question, the requirements for induction of an immune response must be appreciated.

T-cell activation

The primary requirements for T-cell activation are the presentation of processed antigens at the cell surfaces of antigen presenting cells (APC) or target cells, by either class I or class II major histocompatibility complex (MHC) molecules, and the interaction of these antigenic peptide/MHC complexes with T-cell receptors (TCR) of the appropriate antigen specificity [41]. CD8+ T-cells interact with antigen presented by class I MHC molecules and play a major role as cytolytic effector cells. CD4+ T-cells recognize antigen presented by class II MHC, and their major role is to provide T-cell help for B-cells and CD8+ T-cells, although antigen-specific cytolytic CD4+ T-cells have been described [38]. In addition to TCR recognition of antigenic peptide/MHC, T-cell activation requires the interaction of a series of molecules on T-cells with their respective ligands on the APC. The co-stimulatory molecules, B7-1 and B7-2, which interact with the T-cell surface molecules CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) [42, 43], are particularly important. These interactions must occur in concert with engagement of the TCR in order for T-cell activation to occur [44]. Engagement of the TCR in the absence of co-stimulation does not result in T-cell activation but can lead to T-cell paralysis and death [45] (fig. 1). The binding of other molecules, such as the intercellular adhesion molecules (ICAMs), leucocyte function-associated antigen-1 (LFA-1) and others to their ligands on the T-cell helps to stabilize the interaction between the T-cell and the APC, and contributes to T-cell activation [44, 45]. A further requirement is the local release of cytokines, such as interleukin-1 and -2 (IL-1 and IL-2), interferon-gamma (IFN-γ) and others by either APC or T-cells, following the interaction of TCR and co-stimulatory molecules with their respective ligands. These cytokines augment T-cell activation and clonal expansion [45]. Thus, several interactions are necessary in order for an immune response to be generated which will be effective in eliminating a foreign agent or, indeed, a tumour cell.

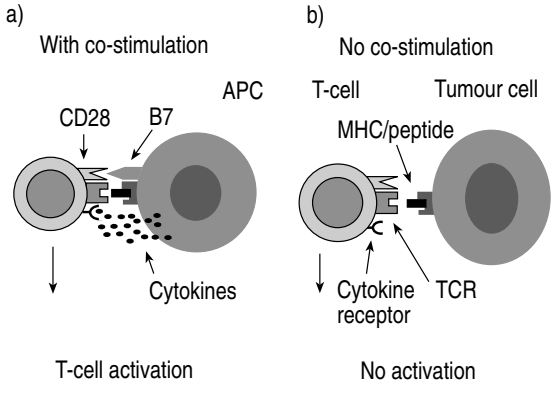

Fig. 1. – A simplified diagram depicting some of the requirements for T-cell activation. a) "Professional" (*e.g.* bone-marrow-derived) antigen presenting cells (APC) express co-stimulatory molecules, such as B7-1, as well as major histocompatibility complex (MHC) molecules, which present antigenic peptides to the T-cell receptor (TCR). Under these circumstances, T-cells can be activated to proliferate, secrete various cytokines and to become cytotoxic. The interaction of certain cytokines, released either by the T-cells themselves, by APC or by neighbouring cells, with appropriate cytokine receptors on the T-cell augments T-cell activation. b) Interaction of the TCR with MHC/peptide in the absence of interaction between co-stimulatory molecules and their cognate receptors, as is most often the case with tumour cells, does not result in T-cell activation but may lead to T-cell anergy.

Table 1. – Mechanisms by which tumours evade immune eradication

1. Downregulation of tumour MHC antigen expression.
2. Production of immunosuppressive factors, *e.g.* TGF-β, prostaglandins.
3. Absence of co-stimulatory molecules leading to induction of anergy.
4. Absence of expression of appropriate target epitopes.
5. Low frequency or affinity of tumour antigen reactive T-cell precursors.
6. Stimulation of specific or nonspecific immunosuppressive cellular activity (macrophages or T-cells).

MHC: major histocompatibility complex; TGF-β: transforming growth factor-β.

Tumour evasion of immune responses

A number of mechanisms have been described which allow tumours to evade immune eradication (table 1). Some of these may be employed in MM. Loss of class I MHC expression is an unlikely candidate, since mesothelioma cells express high levels of class I MHC molecules and this expression can be upregulated with IFN-γ or IFN-α [46, 47]. However, neither human nor murine cell lines constitutively express class II MHC, and in only some lines can this be upregulated with IFN-γ [46]. MM cell lines, like most other tumours, do not generally express the co-stimulatory molecules B7-1 or B7-2 and, at least for the murine cell lines we have examined to date, are relatively devoid of membrane adhesion molecules, such as ICAM-1, although they do express vascular cell adhesion molecule-1 (VCAM-1) on their membrane (Leong, unpublished). Furthermore, MM cell lines have been shown to secrete immunosuppressive factors, such as TGF-β and insulin-like growth factor I (IGF-I) [27, 36, 48, 49], which can inhibit local T-cell activation and cytotoxic activity.

The definition of tumour specific antigens in most tumours (which have not been induced by viruses or chemicals) has been difficult, and very little is known of potential target antigens in MM. The recent demonstration of the expression of simian virus 40 (SV40)-like sequences in a large proportion of human mesotheliomate [50], and the demonstration that many of these patients had circulating antibodies to SV40 antigens does raise the possibility that manipulation of the immune system to mount a cytotoxic response to these antigens may be possible. Recent data from other tumour systems suggest, however, that the antigens which can be recognized by tumour-reactive T-cells are probably not unique to the tumour, but are shared with normal tissues of the same histological origin, or are expressed during differentiation of those tissues [40]. Thus, in order for an effective immune response to be generated, it is not only necessary to overcome the local immunosuppressive milieu and the absence of co-stimulation which may predispose to anergy induction in the tumour environment, but it is probably also necessary to break the pre-existing tolerance to those self-antigens which are the potential targets for tumour-reactive T-cells. Successful generation of an antitumour response against antigens which are expressed both by the tumour and by normal cells of the same histological type has the potential to be manifested as autoimmunity [40].

Enhancement of antitumour immune responses

Several approaches have been employed in clinical trials and in experimental models to try to generate effective tumour-specific antitumour immunity [37, 51, 52]. One approach is the systemic or local application of cytokines as a means of augmenting deficient immune responses, and some of these have been applied to MM. Growth inhibition or lysis of MM cell lines can be effected by a variety of cytokines and immune effector cells. The immunologically relevant cytokines IFN-α, IFN-γ and tumour necrosis factor-α (TNF-α) can directly inhibit the proliferation of some MM cell lines [46, 53–55]. A number of clinical trials have been conducted to assess the therapeutic effect of cytokine treatment of MM. Systemic administration of IFN-α2a produced transient tumour regression in only a fraction of patients [56, 57]. The intrapleural administration of INF-γ to patients with MM at the very earliest stage of disease has been reported to be effective [58], but such early diagnosis is impractical for most centres and most patients. The intrapleural administration of IL-2 was accompanied by unacceptable toxic side-effects [59]. Interestingly, in preclinical studies, a hybrid IFN-α/β was shown to retard the *in vivo* growth of a murine MM cell line, despite having no direct effect on the cell line *in vitro* [60]. These data suggest that the IFN-α/β indirectly affected tumour growth by recruitment of immune effector cells. MM cell lines are susceptible to lysis by a variety of immune effector cells. For example, lymphokine-activated killer (LAK) cells [46, 61], and some T-cell clones bearing γ/δ TCR [62], can lyse MM cells *in vitro* in a non-MHC restricted manner, but natural killer (NK) cells are usually ineffective [46, 61]. Furthermore, specific cytotoxic T-lymphocytes (CTL) can also mediate MM cell lysis (see below).

An alternative approach to cancer immunotherapy has been to genetically modify the tumour cells themselves, so that at least some of the requirements for effective T-cell activation are supplied. Thus, in a number of systems, tumour cells have been transfected with genes encoding either various cytokines [52], self or foreign MHC molecules [63–65], or co-stimulatory molecules [66, 67] in attempts to induce immunity to both the transfectant and the untransfected parental tumour cells. The ultimate aim of these protocols is to generate an immune response which is capable of clearing established tumour cells. The tumour cells will, thus, serve as a source of tumour antigen, and since MM cells express abundant class I MHC it is assumed that epitopes from the target antigen will be presented to T-cells in a milieu more favourable to the generation of an antitumour response.

Enhancement of immunity to MM by gene transfer

We have begun such experiments using a murine model of MM [47]. This model is particularly useful since it

parallels the human disease in terms of growth factor and cytokine expression, MHC expression, histological features, sensitivity to lysis by NK and LAK cells, and because the primary tumours were induced by administration of crocidolite asbestos.

In our initial experiments, we modified a nonimmunogenic MM cell line (AC29) by transfection and expression of allogeneic class I MHC genes. This approach has been reported to bring about rejection of the transfectant and to induce immunity to the parental cell in some experimental systems [64, 68], and is undergoing clinical trial for melanoma and other tumour types [65, 69, 70]. The mechanism of induction of antitumour immunity is unclear, but one possibility is that the generation of a very strong local immune response to the allogeneic MHC causes the release of high local cytokine concentrations, which in turn provide sufficient help to activate bystander T-cells reactive with parental tumour antigens. All of the allo-MHC transfectant MM clones were rejected by the recipient mice, but no immunity to the parental cell line was generated, since it produced tumours in all mice on subsequent challenge [71]. CTL reactive with the allo-MHC molecules could be readily demonstrated, but no tumour-specific CTLs were demonstrable (fig. 2). When parental cells were inoculated together with the allo-MHC transfectants, tumour growth was delayed, however, suggesting some inhibition of parental tumour growth, presumably as a result of the local release of cytokines during the vigorous response to the transfectants.

A second approach which has proven successful in some experimental systems is to modify tumour cells so that they constitutively express class II MHC [72]. The rationale behind this approach is that tumour cells may be able to present endogenous antigen to, and activate, CD4+ T-cells, which in turn will provide help for tumour-reactive CD8+ cytolytic T-cells. AC29, which constitutively expresses class I, but not class II, MHC antigens, was transfected with the genes encoding the syngeneic class II molecule I-A^k (I-$A^k\alpha$, I-$A^k\beta$), and I-A expressing clones were prepared by limiting dilution. These clones grew in the recipient mice as rapidly as the parental cell line, suggesting that at least for this cell line expression of I-A antigen was insufficient to trigger a protective immune response (Leong, unpublished).

Our third approach to tumour modification has been to introduce the gene for the co-stimulatory molecule B7-1 into these cell lines. In some tumour systems, B7-1 transfection has been shown to induce immunity to the transfectant cell line and to generate an immune response which could protect against challenge by the parental unmodified tumour [66]. Some data, however, suggest that B7-1 expression results in protective immunity only for tumours which are immunogenic [67]. Therefore, we have prepared B7-1 expressing transfectant clones from two murine MM cell lines, AC29 (nonimmunogenic) and AB1 (weakly immunogenic). The *in vivo* (but not *in vitro*) growth of three out of four AC29-B7 transfectant clones was delayed compared to control clones, although all four eventually formed tumours in all mice. Of particular interest, however, was the observation that inoculation of the slowest growing B7-1 transfectant (AC29.2.6B7) generated CTL, which were effective against the transfectants as well as the untransfected parental cell line. This was the first demonstration of such a response to this nonimmunogenic tumour (fig. 2). Also of significance was the observation that the fastest growing B7-1 transfectant clone did not generate CTL upon inoculation, although it could be recognized and killed by those CTL generated in response to AC29.2.6B7 (Leong, unpublished). Each of these clones expressed equivalent levels of B7-1 and class I MHC. These data give an indication of the heterogeneous properties of MM, even within a single cell line, but do suggest that if tumour-reactive cytotoxic effector cells can be generated against at least some of the constituent cells within a MM they can be effective against the remainder.

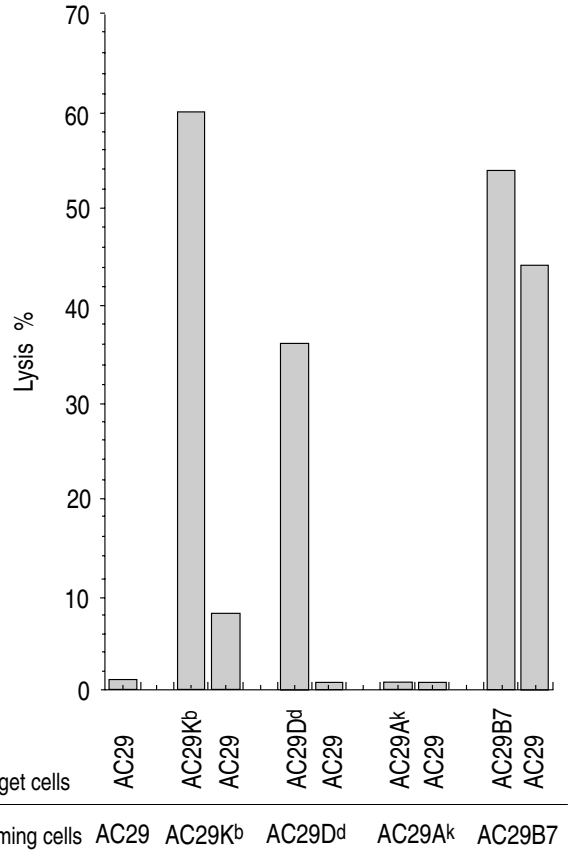

Fig. 2. – Cytotoxic activity of T-cells derived from mice inoculated with live murine malignant mesothelioma (MM) cells. Mice were primed with 1×10^6 parental AC29 MM cells, or with a similar number of each of the transfectant clones indicated. Splenic T-cells from primed mice were then tested for *in vitro* cytotoxic activity against the priming transfectant clone and parental AC29. Priming with either AC29 or the class II MHC (I-A^k) transfectant did not induce cytotoxic T-lymphocytes (CTL). Priming with allogeneic class I MHC transfectants (AC29K^b, AC29D^d) induced CTL capable of killing the priming cells but not the parental tumour cells. Significantly, AC29B7 transfectants induced CTL capable of killing both transfectants and parental AC29, but not other irrelevant tumour cells (not shown). MHC: major histocompatibility complex.

Inoculation of three AB1-B7 transfectants resulted in tumour growth in only 10–70% of recipients, depending on the clone inoculated, although parental AB1 cells

produced tumours in all mice inoculated. Since all transfectant clones expressed similar levels of B7-1, these data suggest clonal heterogeneity within the tumour cell line. Mice which did not develop tumours were rechallenged with parental tumour cells, and further heterogeneity was demonstrated by the fact that the parental tumour did not grow in two of the groups but grew rapidly in the third. Thus, all the transfectant clones were recognizable as targets for the immune system, but only two were able to generate systemic immunity to the parental cell line. As with AC29, inoculation of the AB1-B7 transfectants generated CTL which were effective both against the parent cell line and the transfectants, although untransfected AB1 was unable to do so.

Taken together, these data suggest that the generation of an immune response against MM is a possibility. The cells constitutively express class I MHC molecules which have the potential to present target antigens to MHC restricted CTLs, they are susceptible to lysis by a variety of immune effector cells, and there are indications that growth of a proportion of tumours can be modified by cytokine administration or the inoculation of genetically modified cells. The latter approach holds promise. The challenge will be to provide stimuli which overcome the immunological nonresponsiveness which is contributed to by: 1) the inherent tolerance to the self-antigens which are likely to be target antigens on the tumour cells; 2) the anergy induced by interaction of tumour-reactive T-cells with tumour antigen under suboptimal conditions; and 3) the influence of immunosuppressive factors released by the tumour cells. It is likely that a combination of modifications, perhaps comprising co-stimulatory molecules and cytokines, will be necessary to induce such immunity, particularly given the variations in the effects of genetic modification which have been demonstrated both within and between MM cells lines so far.

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