Expression of B7 co-stimulatory molecules by B16 melanoma results in a natural killer cell-dependent local anti-tumour response, but induces T-cell-dependent systemic immunity only against B7-expressing tumours

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Summary In an attempt to enhance the anti-tumour immune response, the co-stimulatory molecules B7-1 or B7-2 were expressed on the surface of B16 melanoma cells. B7-expressing tumours grew more slowly in both syngeneic immunocompetent mice and athymic T cell-immunodeficient nude mice. The delay in growth of B7-expressing tumours was dependent on natural killer (NK) cells, as reductions in tumour growth rates were minimized in mice depleted of NK cells. Systemic immunity to B16 melanoma was examined by vaccination with irradiated tumour cells. Inoculation with irradiated B16 B7-1 cells failed to protect against a subsequent challenge with live parental B16 cells, but conferred partial protection against challenge with live B16 B7-1 cells. In contrast to the local anti-tumour reaction, this protective response was dependent on T cells. The results presented here reveal some of the mechanisms involved in the in vivo response to a poorly immunogenic tumour modified to express co-stimulatory molecules.

Keywords: melanoma; B7-1; B7-2; co-stimulation; natural killer cell

Attempts to modulate the immune response against tumour cells as a potential therapeutic modality have centred mainly on T cells, as these represent the immune cell population with antigen specificity and memory (Tepper and Mule, 1994; Colomb and Formi, 1996). An effective lytic response against tumour cells requires activation of precytotoxic CD8+ T cells by cytokines secreted from CD4+ T helper cells which, in turn, have been activated by professional antigen-presenting cells (APCs) that have taken up antigens derived from tumour cells (Pardoll, 1993). In addition, professional APCs may also activate CD8+ cytotoxic T cells directly (Huang et al., 1994). Efficient activation of T cells requires antigen-non-specific signals, as well as the antigen-specific signal received by the T-cell receptor/CD3 complex. An important co-stimulatory signal is provided by the CD28 receptor on T cells (Linsley and Ledbetter, 1993), the ligands for which belong to the B7 family, including B7-1 (CD80) (Freeman et al., 1991) and B7-2 (B70/CD86) (Azuma et al., 1993; Freeman et al., 1993). These co-stimulatory molecules are expressed by professional APCs, such as dendritic cells and macrophages, enabling them to present antigens effectively to T cells. Co-stimulatory signals give rise to increased expression of a variety of cytokines that have autocrine and paracrine effects on the proliferation, activation and maturation of T cells (Gimmi et al., 1991).

In recent years, it has become evident that many human and experimental tumours possess specific antigens that may be recognized by T cells and that may act as targets for an immune rejection response (Boon et al., 1994). Such specific antigens have been identified in the B16 murine melanoma (Naftzger et al., 1996; Bloom et al., 1997). However, as most tumour cells do not express co-stimulatory molecules, tumour-specific antigens would fail to be presented to T cells efficiently. Indeed, this may represent one mechanism by which tumour cells evade recognition by the immune system (Vile et al., 1996).

In vitro, activation of cytotoxic T cells may be achieved by stimulation via the TCR and CD28, without requirement for exogenous cytokines provided by T helper cells (Azuma et al., 1992a; Harding and Allison, 1993). In attempts to enhance immune recognition and augment anti-tumour responses, co-stimulatory molecules have been expressed on the surface of tumour cells. The engineered tumour cell would then be capable of presenting the tumour-associated antigens together with the co-stimulatory signal directly to T cells, thereby bypassing the requirement for helper T cells and APCs. B7-expressing tumours in vivo have elicited an effective local anti-tumour response that is mediated by CD8+ cells independent of CD4+ cells. In support of this model, at least in a primary response against the tumour (Chen et al., 1992, Townsend and Allison, 1993). Recently, it has been shown that the in vivo response against some B7-1-expressing tumours is mediated primarily by natural killer (NK) cells, instead of (Geldhof et al., 1995; Yeh et al., 1995), or in addition to, T cells (Cavallo et al., 1995; Wu et al., 1995). Here, we report for the first time that the response against a poorly immunogenic tumour
tumour cell challenge. Although B7-1-expressing B16 cells were ineffective in eliciting a protective response against parental B16 cells, they afforded partial protection against B7-1-expressing B16 cells. Unlike the local reaction against the tumour, this response was T cell dependent. These data raise questions concerning the role of exogenously transferred B7 molecules in the generation of anti-tumour immune responses in vivo.

**MATERIALS AND METHODS**

**Cell culture**

B16 is a long-established murine melanoma cell line (Fidler, 1970). The B16.F1 subline was used in this study. CMT93 is a murine colorectal tumour cell line (Franks and Hemmings, 1978). All cell lines were monitored routinely and found to be free of *Mycoplasma* infection. The cells were cultured in Dulbecco's minimal essential medium supplemented with 10% (vol/vol) fetal calf serum and 4 mM L-glutamine.

**Expression plasmids and transfection of tumour cells**

Subcloning was performed using standard recombinant techniques (Sambrook et al. 1989). Plasmid pTyr-B7-1 is a tissue-specific expression vector where murine B7-1 is driven by the 5' promoter of the murine tyrosinase gene (Vile and Hart, 1993; Chong et al. 1996). The expression vector BCMGNeo-mB70 was kindly provided by Professor M Azuma (Tokyo) (Azuma et al. 1993). Adherent B16 melanoma cells (106) were transfected with 10 μg of plasmid DNA by calcium phosphate precipitation using the Profection method (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cells were selected in 1.25 μg ml-1 puromycin (Sigma, Poole, UK) or in 5 mg ml-1 G418 sulphate (Gibco, Paisley, UK). After incubating in selection medium for 3 weeks, surviving colonies were expanded and assayed for transgene expression. B7-1 was expressed in CMT93 cells using a retroviral vector, as described previously (Chong et al. 1996).

**Flow cytometry**

Expression of B7-1 and B7-2 was determined by staining with the fusion protein mCTLA4-Hyl (Lane et al. 1993) (kindly provided by Dr P Lane, Basle) or the specific monoclonal antibodies 16-10-A1, hamster IgG (anti-B7-1) (Razi-Wolf et al. 1992) (generously given by Dr H Reiser, Boston) or GL1, rat IgG2a (anti-B7-2) (Pharimingen, Cambridge, UK). An appropriate fluorochrome-conjugate secondary antibody was used (all obtained from Dako, Bucks, UK). The samples (5000 cells) were analysed using a Becton Dickinson FACScan.

**In vivo injection of tumour cells**

C57BL/6 mice and BALB/c nude (nu/nu) mice were obtained from colonies bred at the Imperial Cancer Research Fund (Herts, UK). C57 beige mice (C57BL/6Hsd-bgi) were purchased from Harlan (Oxfordshire, United Kingdom). The NK cell activity of the nude mice was higher than that of C57BL/6 mice (data not shown), whereas C57 beige mice were NK cell deficient. Mice were age and sex matched for individual experiments. Institutional guidelines for care and welfare of animals were adhered to strictly. To establish subcutaneous (s.c.) tumours, 1 × 106 cells (experiments involving
Depletion of natural killer cells

In vivo depletion of NK cells was performed by using 25 μl of rabbit polyclonal anti-asialo GM1 (Wako, Neuss, Germany), made up to a volume of 0.2 ml with PBS and injected intravenously (Habu et al. 1981). The animals were challenged with tumour cells 1 day later. Each mouse received a second dose of antibody 1 week later. Control mice were injected with normal rabbit serum.

In vitro assay for natural killer cell activity

The assay was based on lysis of ³⁵Cr-labelled tumour cells by freshly isolated splenocytes (Brunner et al. 1976). Target cells (2 × 10⁶) were incubated with [³⁵Cr]sodium chromate (3.4 MBq) for 1 h, washed and suspended at a concentration of 1 × 10⁶ cells ml⁻¹. Single cell suspensions of spleen cells were prepared. An aliquot of 0.1 ml of the effector and target cell suspensions was mixed at various effector–target ratios, in replicates of at least four, and incubated for 4 h. An aliquot of 0.1 ml of supernatant was aspirated to determine radioactivity. As a positive control in the assays, splenocytes were obtained from mice that had been injected with 100 mg of poly I-poly C (polyinosinic-polycytidylic acid) 24 h previously. This agent is a potent inducer of interferons and rapidly activates NK cell activity (Djeu et al. 1979).

Vaccination of mice with irradiated tumour cells

Tumour cells were suspended in PBS and irradiated (50 Gy). Mice were injected s.c. with 5 × 10⁶ irradiated cells (0.1 ml) in the flank region and two further doses were administered at weekly intervals. The mice received a live cell challenge (5 × 10⁵ B16 or 16 B7-1 cells, 5 × 10⁶ CMT93 B7-1 cells) in the opposite flank 1 week later.

Statistical analyses

Data from the animal experiments were analysed by plotting Kaplan–Meier curves using the ‘occurring event’ as the time at which a tumour appeared. A tumour was considered to be present when a palpable mass > 0.2 cm was noted. Different groups of mice were compared using the log-rank test (Altman. 1991).

RESULTS

Transfection of B7-1 or B7-2 or cDNA into B16 melanoma cells

Parental B16 melanoma cells did not express detectable levels of B7-1 or B7-2 (Figure 1). Stable expression of murine B7-1 was achieved by co-transfection of plasmid pTyr-B7-1 with a plasmid bearing the puromycin-resistance gene (Figure 1). Murine B7-2/B70 was expressed using plasmid BCMGSNeo-mB70 (Figure 1). Expression of these molecules was stable in in vitro culture, up to approximately ten subcultures. The in vitro growth rates of the B7-expressing sublines were similar to the parental line (data not shown).

Expression of the co-stimulatory molecule B7-1 or B7-2 retards the growth rate of B16 melanoma cells in immunocompetent mice

Subcutaneous injection of B16 B7-1 cells into syngeneic immunocompetent C57BL6 mice resulted in the development of tumours...
Expression of B7-1 or B7-2 also retards the growth rate of B16 melanoma cells in athymic T-cell immunodeficient nude mice

A delay in emergence of tumours was also observed when B16 B7-1 melanoma cells were inoculated into athymic T-cell immunodeficient nude mice (P < 0.01, log-rank test) (Figure 2B). Moreover, a number of these mice completely rejected the B7-expressing tumour cells. For instance, 2/10 nude mice injected with B16 B7-1 cells remained tumour-free, whereas all the mice given parental B16 cells developed tumours (Figure 2B). The decreased tumorigenicity of B16 B7-1 cells in nude mice was observed in seven other independently repeated experiments (data not shown), where it was also apparent that the retardation of growth of B16 B7-1 cells in nude mice was more marked than that seen in immunocompetent mice. Expression of B7-2 also decreased the growth rate of B16 tumours in nude mice consistently (P < 0.05, log-rank test) (Figure 3B).

The growth rates of B7-2 and parental tumours were similar in beige mice

No difference was seen in the growth rates of B7-2 and parental tumours in C57 beige mice (Figure 3C). However, a delay in appearance of B7-1 tumours was seen compared with parental tumours (P < 0.01, log-rank test) (Figure 2C).

The difference in growth rates between B7-expressing and parental B16 tumours in nude mice was minimized by treatment with anti-asialo GM1 antibody

Nude mice that were treated with 25 µl of rabbit polyclonal anti-asialo GM1 antibody showed decreased NK cell activity compared with control mice given normal rabbit serum (NRS), as demonstrated by an NK cell lysis assay performed using splenocytes from these mice against ³¹Cr-labelled NK-sensitive YAC cells (data not shown).

When control NRS-treated nude mice were inoculated with parental B16 and B16 B7-1 cells, there was a delay in the emergence of B7-1 tumours (Figure 4A), in keeping with previous results. In contrast, when the same cells were inoculated into anti-asialo GM1-treated nude mice, there was no delay in the appearance of the B7-1 tumours (Figure 4B).

Similar results were seen when B7-2 and parental B16 cells were compared. The growth rate of B16 B7-2 cells was retarded in control NRS-treated nude mice (Figure 4C) but the difference in the B7-2 and parental lines was markedly diminished when compared in anti-asialo GM1-treated mice (Figure 4D).
**B7 induces NK cell-dependent response**

Expression of B7-1 gave rise to systemic protective immunity against B7-1-expressing tumour cells

The ability of tumour cells to elicit systemic protective immunity against parental cells was investigated by vaccination with irradiated tumour cells followed by challenge with live B16 cells. In immunocompetent mice, vaccination with irradiated parental B16 cells did not result in any protection against challenge with live parental B16 cells (Figure 5A). Therefore, under these conditions, B16 tumour cells were poorly immunogenic. Vaccination with irradiated B7-1 cells did not induce any protective immunity against live parental B16 challenge either (Figure 5A). In contrast, vaccination with irradiated B7-1 cells, but not parental B16 cells, consistently elicited partial protection against challenge with live B16 B7-1 cells (P<0.02, log-rank test) (Figure 5B). The majority of mice was completely protected against live cell challenge. These vaccination experiments were also performed in parallel in T-cell-immunodeficient athymic nude mice. In these, however, vaccination with B7-1 cells did not protect against challenge with live B7-1 cells (Figure 6).

Immunocompetent mice were also vaccinated with irradiated B16 B7-1 cells and subsequently challenged with live B7-1-expressing...
DISCUSSION

We have shown here that B16 melanoma cells that express B7-1 or B7-2 grew more slowly than parental B16 cells in immunocompetent syngeneic animals. This retardation in growth was dependent on NK cells, as the B7 tumours also grew more slowly in athymic nude mice, which possess NK cell activity but which are largely deficient in T cells. Also, in nude mice depleted of NK cells, the difference in growth rates between the parental and B7 tumours was minimal. Similarly, in NK cell-deficient beige mice, at least in the case of the B7-2 tumours, the growth rates between parental and gene-modified B16 cells did not differ. These results show clearly the involvement of NK cells in the response against B7-expressing tumours, although they do not exclude the participation of other effector cell types, as is suggested by the delayed growth of B7-1 tumours in beige mice.

Natural killer cells represent a heterogeneous population of lymphocytes that possess spontaneous cytolytic activity against some tumour cells and, unlike T cells, they are unrestricted by antigen specificity (Whiteside and Herberman, 1995). NK cells also secrete a variety of cytokines that contribute to the activation of other cells, including T cells. It has been presumed that NK cells play a role in the response against tumours in vivo as they can eliminate circulating tumour cells (Riccardi et al. 1980; Barlozzari et al. 1983). Recently, a subpopulation of NK cells was identified that is able to extravasate, migrate into solid tumour tissue and function as cytolytic cells therein (Vujanovic et al. 1995). Also, of late, there has been considerable progress in the definition of receptors on NK cells that regulate their activity (Lanier, 1997). A family of receptors recognizes MHC class I molecules and generates an inhibitory signal to NK cells. In the mouse these belong to the Ly49 family of proteins, whereas in humans this function is provided by the family of killer cell inhibitory receptors (KIR) (Raulet, 1996). Low expression of MHC class I by tumours such as B16 melanoma leads to poor recognition by T cells, but may favour NK cell activation as MHC class I interacts with these inhibitory molecules.

The stimulatory receptors on NK cells are not as well understood, but a variety of different molecules are assumed to be responsible for this activity. One family of receptors with stimulatory activity is NKR-P1, which recognizes oligosaccharide moieties on tumour cells (Bezouska et al. 1994). CD28, which is expressed on murine NK cells, also provides a stimulatory signal on binding with B7, promoting NK cell proliferation and increased cytokine secretion (Nandi et al. 1994). Recently, B7-1 was reported to bind to an unidentified receptor on murine NK cells and trigger cytolytic activity (Chambers et al. 1996). Moreover, this signal was able to override the inhibitory signals imparted by MHC class I molecules. Human NK cell lines derived from the YT line also express CD28, and the cytolytic activity of these cell lines is enhanced by the interaction of CD28 with B7 (Azuma et al. 1992b; Montel et al. 1995). Moreover, fresh human NK cells have been found to lyse B7-1-expressing tumour cells preferentially (Dessureault and Gallinger, 1996).

Other groups have described varied effects of B7-1 expression on in vivo growth of B16 melanoma. In some cases, B7-1 did not affect the growth pattern (Chen et al. 1994; Townsend et al. 1994), whereas in others it resulted in complete rejection of B16 tumours, a response that was mediated by NK cells and CD8 cells (Wu et al. 1995). These apparently conflicting results probably reflect differing levels of B7-1 expression, as it has been reported that only clones expressing high levels of B7-1 were rejected completely, whereas low-expressing clones grew more slowly but were not rejected (Wu et al. 1995). The results we present here are compatible with this possibility. The clone used in our experiments expressed a comparable level of B7-1 to the low-expressing clone in the latter study. Under in vitro conditions, B7-expressing tumour cells were no more susceptible to lysis by fresh splenocytes than were the parental cells (data not shown). This does not correlate with the in vivo results, but may simply reflect the less than ideal nature of the in vitro assay.

As well as the effects on local tumour growth, it is also of importance to determine the effects of B7 co-stimulatory molecules on systemic immunity. In some studies, B7-expressing tumours have generated protective immunity against a subsequent challenge with parental tumour cells (Cavallo et al. 1995; Gajewski et al. 1996; Dunussi-Joannopoulos et al. 1996) although in other studies such tumours did not elicit any protective immunity (Ramartnannam et al. 1994; Katsanis et al. 1995). Previously, in the moderately immunogenic tumour model (CI735 murine melanoma) we demonstrated that B7-expressing tumours may even reduce the degree of systemic immunity relative to that elicited by the parental tumour (Chong et al. 1996). In contrast, B16 melanoma is a tumour of low intrinsic immunogenicity.

Figure 6 Lack of systemic protective immunity in T-cell immunodeficient athymic nude mice following vaccination with irradiated parental B16 and B16 B7-1 cells. BALB/c nude mice were vaccinated with 5 x 10⁶ irradiated parental or B7-1 cells, as indicated (seven or eight mice per group) (three vaccinations at weekly intervals). One week later, the mice were challenged with 5 x 10⁶ live (A) parental cells or (B) B7-1 cells. The figure shows the growth of tumours arising from the live cell challenge.

CMT93 cells, an unrelated syngeneic tumour cell line (Chong et al. 1996). Most of these mice (six of seven) did not develop tumours following challenge with CMT93 B7-1 cells and remained tumour free (Figure 5C). In comparison, mice vaccinated with irradiated parental B16 cells and unvaccinated mice developed tumours more readily, although the difference compared with the group vaccinated with B16 B7-1 cells did not reach statistical significance.
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