

Induction of an antitumour adaptive immune response elicited by tumour cells expressing *de novo* B7-1 mainly depends on the anatomical site of their delivery: the dose applied regulates the expansion of the response

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SUMMARY

De novo expression of costimulatory molecules in tumours generally increases their immunogenicity, but does not always induce a protective response against the parental tumour. This issue was addressed in the mouse Sp6 hybridoma model, comparing different immunization routes (subcutaneous, intraperitoneal and intravenous) and doses (0.5×10^6 and 5×10^6 cells) of Sp6 cells expressing *de novo* B7-1 (Sp6/B7). The results can be summarized as follows. First, *de novo* expression of B7-1 rendered Sp6 immunogenic, as it significantly reduced the tumour incidence to $\leq 15\%$ with all delivery routes and doses tested, whereas wild-type Sp6 was invariably tumorigenic (100% tumour incidence). Second, long-lasting protection against wild-type Sp6 was mainly achieved when immunization with Sp6/B7 was subcutaneous: a dose of 0.5×10^6 Sp6/B7 cells elicited protection that was confined to sites in the same anatomical quarter as the immunizing injection. Repeated injections of the same dose extended protection against wild-type Sp6 to other anatomical districts, as well as a single injection of a 10-fold higher dose (5×10^6 cells). Finally, Sp6-specific cytotoxic T-lymphocyte activity was detected in draining lymph nodes, and the splenic expansion of Sp6-specific cytotoxic T-lymphocyte precursors quantitatively correlated with the dose of antigen. We conclude that activation of a protective immune response against Sp6 depends on the local environment where the immunogenic form of the 'whole tumour cell antigen' is delivered. The antigen dose regulates the anatomical extent of the protective response.

INTRODUCTION

Tumours are often poorly immunogenic, as they mostly express antigens belonging to self or minimally altered self and may adopt different strategies to evade immune surveillance, such as

secretion of immunosuppressive factors (i.e. transforming growth factor- β , prostaglandins) and/or modulation of receptors. This may result in the induction of tolerance/anergy of effector cells.¹ In animal models, immunization against tumours has been successfully obtained, even without any knowledge of the specific tumour antigens involved, by using tumour cells genetically engineered to express *de novo* cytokines or major histocompatibility complex (MHC) molecules as 'whole tumour cell antigen'.^{2,3} *De novo* expression of B7-1 and B7-2 costimulatory molecules (namely CD80 and CD86) has also been shown to increase tumour immunogenicity.^{4–6} Primary rejection of B7-modified tumour cells has been shown to involve a complex effector population, consisting of natural killer (NK) and NK T cells, granulocytes and CD8⁺ T cells.^{7–9} Direct priming of CD8⁺ T-cell effectors has also been demonstrated.^{10–13} Unfortunately, the improved immunogenicity mediated by *de novo* expression of B7 does not necessarily result in the rejection of unmodified parental tumour cells given

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Abbreviations: CTL, cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; i.p., intraperitoneal; i.s., intrasplenic; i.v., intravenous; MHC, major histocompatibility complex; s.c., subcutaneous; Sp6/B7, Sp6 transfected with B7-1; Sp6/pSR α -neo, Sp6 transfected with pSR α -neo; WT, wild type.

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in successive challenges.^{8,9,13} Indeed, immunogenicity is a necessary prerequisite, but not sufficient, to trigger an effective immune response: the dose of antigen associated with the anatomical site and the time schedule of antigen delivery may be as crucial as immunogenicity.^{14–16}

In order to be recognized by naive lymphocytes and initiate a specific immune response, antigens must reach secondary lymphoid organs.¹⁷ Thus, the anatomical site of delivery of the immunogen might be expected to play a crucial role. The quantity of tumour cells in the inoculum may well determine the persistence of their antigens in the lymphoid organs and thus influence the level of expansion of tumour-specific T-cell clones.^{14–16} The efficacy of an antitumour response may also depend on the dynamic ratio of tumour growth at different anatomical locations to the cytotoxic T-lymphocyte (CTL) response.¹⁷

Thus, besides improving tumour immunogenicity, the three parameters of antigen dose, site of delivery and time schedule of immunization, should also be considered in order to obtain an optimal immune response. All these aspects have been investigated *in vivo* using the Sp6 hybridoma as a tumour model in the syngeneic BALB/c mouse.¹⁸ Wild-type (WT) Sp6 cells give rise to tumours in 100% of cases, after injection of varying amounts of cells via different administration routes, namely subcutaneously (s.c.), intraperitoneally (i.p.), intravenously (i.v.) and intrasplenically (i.s.). However, *de novo* expression of the B7-1 costimulatory molecule, obtained by stable transfection of Sp6 with specific cDNA, completely inhibited tumour growth in immunocompetent mice. Rejection of WT Sp6 was triggered by immunization with B7-1-transfected Sp6 cells almost exclusively via the s.c. route, in a dose- and time-dependent response. Worthy of note was the correlation found between the anatomical extent of tumour protection and expansion of tumour-specific CTL precursors.

MATERIALS AND METHODS

Cell lines and transfections

Sp6 hybridoma cells, syngeneic with the BALB/c mouse strain (H-2^d genotype), were chosen for the present work in view of their ability to be transfected and to maintain the transfected genes in a permanent, integrated form.¹⁸ Sp6 cells were transfected with the full-length mouse B7-1 cDNA, kindly donated by Dr Giulia Casorati and Dr Paolo Dellabona (Unità d'Immunochimica, DIBIT, Istituto Scientifico San Raffaele, Milan, Italy), subcloned into the eukaryotic expression vector, pSR α -Neo, containing the G418 resistance gene⁷ and with the plasmid vector, pSR α -Neo, without inserts (Invitrogen Corp., San Diego, CA). Transfections were performed by electroporation with a Bio-Rad apparatus (Life Science Segrate (MI), Italy) using 5 μ g of DNA added to 4 $\times$ 10⁶ cells resuspended in complete medium in 0.2-mm cuvettes, at 200 V, 250 μ F. Selection of transfectants was carried out by growing cells in the presence of 500 μ g/ml G418 (Geneticin G418 sulphate; Invitrogen Corp.), and by immunofluorescence analysis for *de novo* expression of B7-1. Transfectants were indicated as follows: Sp6/pSR α -Neo (Sp6 transfected with the pSR α -Neo empty vector); and Sp6/B7 (Sp6 transfected with pSR α -Neo/B7), expressing *de novo* B7-1 the costimulatory molecule.

Immunofluorescence analysis and monoclonal antibodies

Cells were analysed by indirect immunofluorescence and cytofluorimetry on an Epics XL apparatus (Coulter, Hialeah, FL) using the following monoclonal antibodies (mAbs): 34.1.2S, specific for mouse H-2K^d and D^d MHC class I molecules; 28.14.8S, specific for mouse H2-L^d MHC class I molecules; 25.9.17, specific for mouse I-A^{b,d} MHC class II molecules; K22.42.2, specific for mouse I-E^{b,d} MHC class II molecules; 16-10A1, specific for the mouse B7-1 (CD80) costimulatory molecule; GL-1, specific for the mouse B7-2 (CD86) costimulatory molecule; and BE29G1, specific for the mouse intercellular adhesion molecule-1 (ICAM-1). The secondary antibodies used were goat anti-mouse immunoglobulin G (IgG), fluorescein isothiocyanate (FITC)-IgG F(ab')₂ conjugate (Instrumentation Laboratory, Milan, Italy) for anti-MHC; mouse, anti-hamster IgG, FITC-IgG conjugate for anti-B7-1; and goat anti-rat IgG, FITC-IgG conjugate for anti B7-2 and anti-ICAM-1 primary antibodies (BD-Pharmingen, Milan, Italy).

Growth assays with WT and transfected tumour cells

Growth assays with oligoclonal populations of WT and transfected tumour cells were carried out by comparing cell counts in a cytofluorimeter (Epics XL; Coulter), every 8 hr, as described previously.¹⁹

In vivo experiments

Three-month-old males and/or females of the BALB/c strain of mice [BALB/cByJlco; Charles River Italia, Calco (LC) Italy], syngeneic with Sp6 cells (H-2^d), were injected s.c., i.p. or i.v. with 0.5 $\times$ 10⁶ WT Sp6, Sp6/B7 or Sp6/pSR α -Neo cells resuspended in 0.2–0.5 ml of phosphate-buffered saline (PBS), and s.c. or i.p. with 5 $\times$ 10⁶ cells resuspended in 0.5 ml of PBS. The i.s. injections were performed as described previously,²⁰ with 2 $\times$ 10⁶ cells resuspended in 0.3 ml of PBS.

WT Sp6 and transfectants were also injected s.c., i.p. or i.v. in nude mice of the BALB/c strain (BALB/cByJlco-nu/nu; Charles River Italia) (0.5 $\times$ 10⁶ cells).

All animals were killed as soon as signs of pain and fatigue were perceived. All *in vivo* experiments were approved by the Italian Ministry of Health (Ministerial Decree no. 46/2000-B).

In vitro cytotoxicity assay

The cytotoxicity of spleen- and lymph node-derived cells from naive and treated mice was measured in a 4-hr ⁵¹Cr-standard release assay. Owing to the very low cytotoxic activity elicited in the initial steps of the immunization protocol, a 100 : 1 effector/target ratio was chosen as the reference ratio for all the cytotoxicity experiments performed. In experiments using cells from draining and contralateral lymph nodes, seven tumour-free animals were injected s.c. with Sp6/B7 on one side of the ventral surface in the lower flank region. The draining lymph node was the inguinal one at the site of injection. The non-draining lymph node used for comparison was the contralateral inguinal one. This experiment was performed three times.

T-cell subsets of CD8⁺ and CD4⁺ phenotypes were purified from the spleen of injected animals with magnetic cell sorting using MACS CD8a (Ly-2) and CD4 (L3T4) MicroBeads (Miltenyi Biotec, Bergish Gladbach, Germany), according to the manufacturer's instructions.

Statistics

The tumour incidence values between different treatment groups were compared using the χ^2 -test, with the Yates correction.

RESULTS

Transfection of B7-1 cDNA induced expression of the encoded molecule on the cell surface and did not alter the growth kinetics of Sp6 tumour cells *in vitro*

As shown in Fig. 1, WT Sp6 expressed the MHC class I H2-K and H2-D molecules and ICAM-1, but not the MHC class II, B7-1 and B7-2 molecules. The expression of the H2-L-MHC class I molecular subset was mostly negligible. Transfection of Sp6 with B7-1 cDNA allowed *de novo* expression of B7-1, without altering the expression of MHC class I and class II, B7-2 or ICAM-1. The expression of ICAM-1 was monitored because of its relevance in T-cell activation and tumour immunity.^{7,21} Only the Sp6/B7 transfectants, expressing high levels of B7-1 molecules, were stable over time in culture and could therefore be used for the experiments described here.

To exclude tumour growth alterations caused by transfection, the growth kinetics of WT Sp6 and transfectants were compared *in vitro* using a sensitive, reproducible cytofluorimetric technique.¹⁹ No significant differences in growth kinetics were observed for WT and transfected tumour cells (data not shown).

De novo expression of B7-1 significantly reduced tumour incidence in syngeneic, immunocompetent animals

Animals injected with Sp6/B7 displayed a statistically significant reduction in tumour incidence in comparison to those injected with WT Sp6 and Sp6/pSR α neo. The tumour incidence of WT Sp6 and Sp6/pSR α neo was 100% with all injection routes and doses employed (data not shown), whilst that of Sp6/B7 varied from 0 to 15% ($P < 0.001$) (see Tables 1 and 2). All tumours were detected within 2–3 weeks of injection. Sp6/B7-injected animals that did not develop tumours within this time-period remained tumour-free at autopsy, as found by monitoring three groups of eight to 10 animals, each injected with Sp6/B7 sc., i.p. or i.v. (0.5×10^6 cells), up to 1 year after injection with Sp6/B7 (data not shown).

None of the tumours developing in mice injected with Sp6/B7 expressed B7-1, as determined by cytofluorimetric analysis, because of deletion of B7-1-coding cDNA, as assessed by polymerase chain reaction (PCR) (data not shown). Similar findings have also been described for other mouse tumour systems.^{10,22}

When Sp6/B7 cells were injected in nude mice of the BALB/c strain, 85% of the animals developed B7-1-positive tumours (data not shown). Injection of WT Sp6 in nude mice gave the same results as in immunocompetent mice, namely, 100% tumour incidence (data not shown).

These data suggest that the expression of B7-1 rendered Sp6 immunogenic and susceptible mainly to T-cell effector mechanisms of the adaptive immune response. Effector mechanisms of

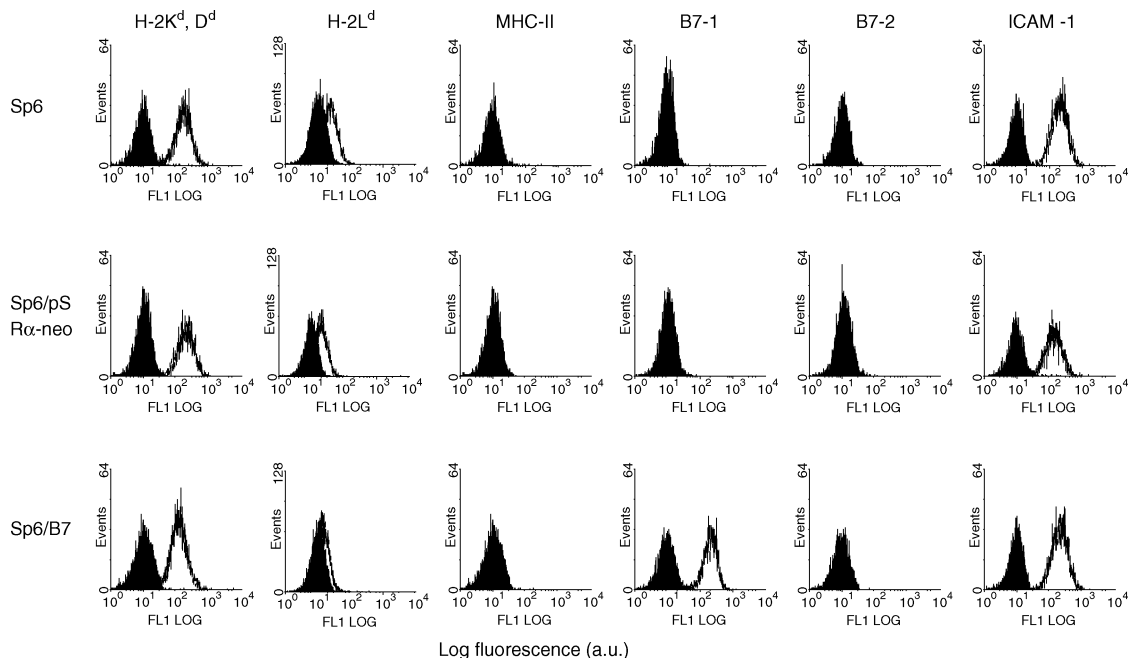


Figure 1. Cytofluorimetric analysis of Sp6, Sp6/pSR α -neo and Sp6/B7 cells after indirect immunofluorescence staining with monoclonal antibodies specific for the mouse major histocompatibility complex (MHC) class I molecules H-2K, D and L, the MHC class II molecules I-A and I-E, the B7-1 and B7-2 costimulatory molecules, and the intercellular adhesion molecule-1 (ICAM-1), as indicated at the top of each lane. The different cells are indicated to the left of each horizontal series of histograms. Black histograms indicate the fluorescence of each sample treated with the secondary monoclonal antibody only. Fluorescence values are expressed in absorbance units (a.u.).

Table 1. Tumour incidence in tumour-free animals injected with 0.5×10^6 Sp6/B7 cells and challenged with wild-type (WT) Sp6 (0.5×10^6 cells) after at least 3 weeks

First injection with Sp6/B7	Challenge with WT Sp6§		
	s.c.	i.p.	i.v.
s.c.			
21/188* (11%)	13/56 (23%)*† 10/19 (53%)*‡	10/10 (100%)	10/10 (100%)
i.p.			
13/85 (15%)	10/10 (100%)	10/10 (100%)	10/10 (100%)
i.v.			
7/62 (11%)	10/10 (100%)	10/10 (100%)	10/10 (100%)

*Tumour incidence is indicated as the number of animals developing tumours/total number of animals injected and as a percentage of animals developing tumours (in parenthesis).

†Results of the second subcutaneous (s.c.) injection given locally relative to the first, within a radius of 0.5 cm around the first inoculum (in the same anatomical quarter).

‡Results of the second s.c. injection given in a different anatomical quarter.

§The differences in tumour incidence between challenge of WT Sp6 s.c. in the same anatomical quarter as the first and challenge of WT Sp6 s.c. in a different anatomical quarter are statistically significant ($P < 0.05$) as well as those between s.c. and intraperitoneal (i.p.) or intravenous (i.v.) challenges (s.c. locally relative to the first versus i.p. and i.v., $P < 0.001$; s.c. in a different anatomical quarter versus i.p. and i.v., $P < 0.05$).

Table 2. Tumour incidence in mice injected subcutaneously (s.c.) and intraperitoneally (i.p.) with 5×10^6 Sp6/B7 cells, intrasplenically (i.s.) with 2×10^6 Sp6/B7 cells, and challenged i.p. and s.c. with 0.5×10^6 wild-type (WT) Sp6 cells after 3 weeks

First injection with Sp6/B7	Challenge with WT Sp6	
	s.c.	i.p.
s.c.		
4/60 (7%)	2/10 (20%)*† 5/10 (50%)*‡§	4/19 (21%)*¶
i.p.		
1/15 (7%)	7/9 (78%)	10/14 (71%)
i.s.		
0/5 (0%)	5/5 (100%)	ND

*The tumour incidence is indicated as the number of animals developing tumours/total number of animals injected and as the percentage of animals developing tumours (in parenthesis).

†Results of the second s.c. injection are given locally relative to the first, within a radius of 0.5 cm around the first inoculum (same anatomical quarter).

‡Results of the second s.c. injection, which was given in a different anatomical quarter.

§The difference in tumour incidence between animals treated with the first s.c. injection and s.c. challenge (given in a different anatomical quarter) and animals treated with all the other schedules, as well as the difference in tumour incidence between animals treated with the first s.c. injection and the s.c. challenge given in the same anatomical quarter and animals treated with the first s.c. injection and the s.c. challenge in a different anatomical quarter, are not significant.

¶The difference in tumour incidence between animals treated with the first s.c. injection, i.p. challenge and animals treated with the first i.p. or i.s. injection is statistically significant ($P < 0.025$). The difference in tumour incidence between animals treated with the first s.c. injection and s.c. challenge (given locally relative to the first, within a radius of 0.5 cm around the first inoculum) and the first i.p. injection and s.c. challenge is statistically significant ($P < 0.01$).

the innate immune response would appear to play only a minor role.

Tumour-free animals injected with 0.5×10^6 Sp6/B7 cells s.c. display localized protection against the challenge with WT Sp6 tumour: Sp6-specific cytotoxic activity is found in the spleen and draining lymph nodes

On subjecting tumour-free animals, immunized with Sp6/B7 s.c., to challenges with WT Sp6 s.c. at different time-points, we found that statistically significant protection starts 3 weeks after immunization and remains constant up to at least 8 weeks (data not shown).

Tumour-free animals, following injection with 0.5×10^6 Sp6/B7 cells s.c., i.p. or i.v., were challenged with WT Sp6 (0.5×10^6 cells) 3 weeks after immunization. As shown in Table 1, only mice injected s.c. with Sp6/B7 cells were protected against challenge with WT Sp6, provided that it was given s.c. The protection was stronger when the challenge was given in the same anatomical quarter (usually within a radius of 0.5 cm around the first Sp6/B7 inoculum) (23% tumour incidence) than when it was given in a different anatomical quarter (whether contralateral or ipsilateral) (53% tumour incidence). These differences were statistically significant ($P < 0.05$) (Table 1). When challenged with WT Sp6 cells i.p. or i.v., the same animals were not protected at all (100% tumour incidence, as shown in Table 1). Finally, i.p. and i.v. injections of 0.5×10^6 Sp6/B7 did not protect against challenge with WT Sp6, whether given s.c., i.p. or i.v. (100% tumour incidence, as shown in Table 1).

Spleen cells from mice injected s.c. and i.p. with Sp6/B7 showed comparable cytotoxic activity against both Sp6 and Sp6/B7 targets (Fig. 2, histogram i.p. -0.5 and histogram 1 s.c. -0.5). Spleen cells from naive mice showed negligible levels of cytotoxicity against the same targets (Fig. 2). Spleen cells from i.v.-injected mice showed low, but by no means negligible, cytotoxicity against the Sp6/B7 target only (Fig. 2, histogram i.v. -0.5). In mice injected s.c. with Sp6/B7, cells from draining, but not contralateral, lymph nodes showed Sp6/B7-Sp6-specific cytotoxic activity, which was, however, lower than that of spleen-derived cells (Fig. 3). Cells from mesenteric lymph nodes of mice injected s.c., i.p. and i.v. showed the same negligible levels of cytotoxic activity found in naive mice (data not shown).

Repeated injections of the 0.5×10^6 Sp6/B7 cell dose extend the protection against WT Sp6 to anatomically distant sites, which is accompanied with an increase in Sp6-specific CTL precursors in the spleen

As it has been postulated that repeated injections of B7-positive tumour cells could expand tumour immunity,^{23,24} mice surviving s.c. challenge with 0.5×10^6 WT Sp6 cells were rechallenged with 0.5×10^6 WT Sp6 cells s.c. and i.p. 3 weeks later. Full protection (0% tumour incidence) against i.p. challenge and subsequently against i.v. challenge with WT Sp6 (0.5×10^6 cells) was achieved after three s.c. injections (first injection: Sp6/B7, second and third: WT Sp6), always given in the same anatomical quarter and at time intervals of 3 weeks, as

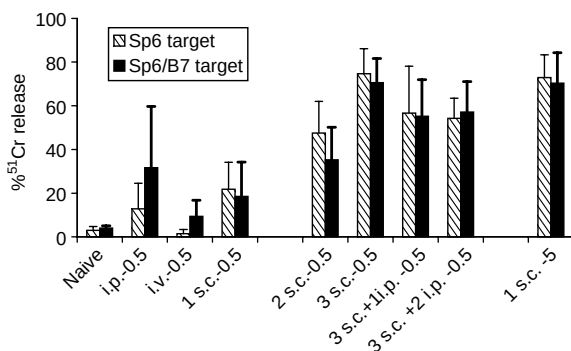


Figure 2. Cytotoxic response against wild-type (WT) Sp6 and Sp6/B7 of cells from spleens of mice immunized with 0.5×10^6 and 5×10^6 Sp6/B7 and/or WT Sp6 cells, expressed as % ^{51}Cr release. Each histogram indicates the mean value with standard deviation (SD) of the results relative to the spleen cells from at least five animals from each group of differently injected mice. Black histograms show the percentage lysis of Sp6/B7 targets, and striped histograms the percentage lysis of WT Sp6 targets. As indicated in the Materials and methods, all experiments were performed with a 100 : 1 effector/target ratio. The type of treatment for each group of mice is indicated on the abscissa: naïve, untreated mice; i.p. -0.5, mice injected once with 0.5×10^6 Sp6/B7 cells intraperitoneally; i.v. -0.5, mice injected once with 0.5×10^6 Sp6/B7 cells intravenously; 1 s.c. -0.5, mice injected once with Sp6/B7 cells subcutaneously; 2 s.c. -0.5, mice injected once with 0.5×10^6 Sp6/B7 cells s.c., then a second time with Sp6/B7 or WT Sp6 cells s.c. (0.5×10^6 cells); 3 s.c. -0.5, mice injected once with Sp6/B7 s.c., then twice with Sp6/B7 or WT Sp6 s.c. (0.5×10^6 cells); 3 s.c. + 1 i.p. -0.5, mice injected once with Sp6/B7 s.c., twice with Sp6/B7 or WT Sp6 s.c. and then once with WT Sp6 i.p. (0.5×10^6 cells); 3 s.c. + 2 i.p. -0.5, mice injected once with Sp6/B7 s.c., twice with Sp6/B7 or WT Sp6 s.c. and then twice with WT Sp6 i.p. (0.5×10^6 cells); 1 s.c. -5, mice injected once with 5×10^6 Sp6/B7 cells s.c. Second and third s.c. injections were given locally relative to the first, within a radius of 0.5 cm around the first inoculum, i.e. in the same anatomical quarter.

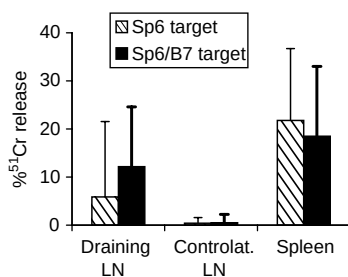


Figure 3. Cytotoxic response against wild-type (WT) Sp6 and Sp6/B7 of cells from draining lymph nodes, contralateral lymph nodes and spleens of mice injected s.c. with 0.5×10^6 Sp6/B7 cells, expressed as % ^{51}Cr release. Each histogram indicates the mean value and standard deviation of three separate experiments with pools of cells from draining lymph nodes, contralateral lymph nodes and spleens from seven different individuals. The origin of the effector cells from each group of histograms is indicated on the abscissa. Black histograms show the percentage lysis of Sp6/B7 targets, and striped histograms the percentage lysis of WT Sp6 targets. As indicated in the Materials and methods, all experiments were performed with a 100 : 1 effector/target ratio.

Table 3. Tumour incidence after repeated injections of 0.5×10^6 Sp6/B7-Sp6 cells given every 3 weeks

First injection s.c.*	Second injection s.c.†	Third injection s.c.†	Fourth injection i.p.	Fifth injection i.v.
Sp6/B7	WT Sp6	WT Sp6	WT Sp6	WT Sp6
21/188‡ (11%)	13/56 (23%)	2/27 (7%)	0/20 (0%)	0/15 (0%)

*The first subcutaneous (s.c.) injection was given in the right or the left shoulder.

†The second and third s.c. injections were given locally relative to the first, within a radius of 0.5 cm around the first inoculum (same anatomical quarter).

‡Tumour incidence is indicated as the number of animals developing tumours/total number of animals injected and as a percentage of animals developing tumours (in parenthesis).

WT, wild type.

shown in Table 3. Protection against i.p. challenge was only partial following two s.c. injections (first injection: Sp6/B7, second: WT Sp6), with a tumour incidence of four in 12 animals (33%) (data not shown). When all three successive s.c. injections were performed with Sp6/B7 cells, 20% of the mice still developed tumours when challenged with WT Sp6 i.p. (Table 4). Three successive injections with Sp6/B7 i.p. afforded much lower protection against i.p. challenge with WT Sp6 (57% tumour incidence) than s.c. injections (20% tumour incidence) ($P < 0.01$ when comparing the protection afforded by three s.c. injections versus three i.p. injections) (Table 4). In contrast, three successive i.v. injections afforded no protection at all against challenges with WT Sp6 i.p. and i.v. (100% tumour incidence) (Table 4 and data not shown).

The cytotoxic activities of spleen-derived cells from mice immunized with serial injections of the 0.5×10^6 cell dose were compared. As shown in Fig. 2 (compare histogram

Table 4. Tumour incidence in mice treated with three repeated injections of 0.5×10^6 Sp6/B7 cells subcutaneously (s.c.), intraperitoneally (i.p.) or intravenously (i.v.), given at time intervals of 3 weeks and challenged i.p. with 0.5×10^6 wild-type (WT) Sp6 cells

Mode of injection of Sp6/B7	Tumour incidence after each injection of Sp6/B7			
	First injection	Second injection	Third injection	Challenge with WT Sp6
s.c.	21/188* (11%)	0/20† (0%)	0/20† (0%)	4/20‡ (20%)
i.p.	11/75 (15%)	0/28 (0%)	0/28 (0%)	16/28‡ (57%)
i.v.	7/62 (11%)	0/10 (0%)	0/10 (0%)	5/5‡ (100%)

*The tumour incidence is indicated as the number of animals developing tumours/total number of animals injected and as the percentage of animals developing tumours (in parenthesis).

†The second and third s.c. injections were given locally relative to the first, within a radius of 0.5 cm around the first inoculum (same anatomical quarter).

‡The difference in tumour incidence after i.p. challenge of wild-type (WT) Sp6 between s.c. and i.p. immunization with Sp6/B7 was statistically significant ($P < 0.05$), as was that between s.c. and i.v. immunization ($P < 0.001$).

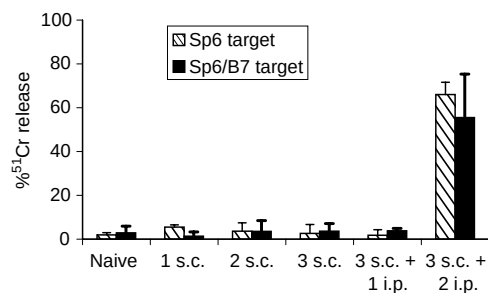


Figure 4. Cytotoxic response against wild-type (WT) Sp6 and Sp6/B7 of cells from mesenteric lymph nodes of mice treated a variable number of times with 0.5×10^6 Sp6/B7-WT Sp6 cells, expressed as % ⁵¹Cr release. Each histogram indicates the mean value plus standard deviation of the results from mesenteric lymph node cells from at least five animals in each group of mice. Black histograms show the percentage lysis of Sp6/B7 targets, and striped histograms the percentage lysis of WT Sp6 targets. As indicated in the Materials and methods, all experiments were performed with a 100 : 1 effector/target ratio. The type of treatment for each group of mice is indicated on the abscissa: naïve, untreated mice; 1 s.c., mice injected once with Sp6/B7 cells subcutaneously; 2 s.c., mice injected once with Sp6/B7 cells s.c., then a second time with Sp6/B7 or WT Sp6 cells s.c.; 3 s.c., mice injected once with Sp6/B7 s.c., then twice with Sp6/B7 or WT Sp6 s.c.; 3 s.c. + 1 i.p., mice injected once with Sp6/B7 s.c., twice with Sp6/B7 or WT Sp6 s.c. and then once with WT Sp6 i.p.; 3 s.c. + 2 i.p., mice injected once with Sp6/B7 s.c., twice with Sp6/B7 or WT Sp6 s.c. and then twice with WT Sp6 i.p. Second and third s.c. injections were given locally relative to the first, within a radius of 0.5 cm around the first inoculum. The cytotoxic activity of mesenteric lymph nodes from mice injected with 5×10^6 Sp6/B7 s.c., and then twice i.p. with 0.5×10^6 WT Sp6 cells, was very similar to that of mice injected once with Sp6/B7 s.c., twice with Sp6/B7 or WT Sp6 s.c. and then twice with WT Sp6 i.p., shown here as 3 s.c. + 2 i.p.

1 s.c. -0.5 with histogram 2 s.c. -0.5 and histogram 3 s.c. -0.5), Sp6 and Sp6/B7-specific cytotoxic activity of splenocytes rapidly increased with the number of s.c. injections, reaching a plateau after the third inoculum (see histogram 3 s.c. -0.5, histogram 3 s.c. +1 i.p. -0.5, and histogram 3 s.c. + 2 i.p. -0.5, in Fig. 2). In contrast, the Sp6 and Sp6/B7-specific cytotoxic activity of mesenteric and inguinal lymph nodes increased only after the second i.p. injection (Fig. 4).

Immunization with a dose of 5×10^6 Sp6/B7 cells s.c. induces the same levels of protection against i.p. challenge with WT Sp6 observed with three successive s.c. injections of a 10-fold lower dose

Naive BALB/c mice were also injected s.c. and i.p. with 5×10^6 Sp6/B7 cells, this dosage being 10-fold higher than that used in the experiments described above. Three weeks later, tumour-free animals were challenged s.c. and i.p. with 0.5×10^6 WT Sp6 cells. As shown in Table 2, s.c. immunization with 5×10^6 Sp6/B7 cells induced protection against an i.p. challenge with 0.5×10^6 WT Sp6 cells which was similar to that obtained with three successive injections of 10-fold fewer Sp6/B7 cells (20% tumour incidence) (Table 4). A further increase in the s.c. Sp6/B7 immunizing inoculum (10×10^6

cells) did not improve the protection (data not shown). There was also no increase in protection against s.c. challenge with 0.5×10^6 Sp6 cells in a different anatomical quarter (see Tables 1 and 2).

The Sp6-specific cytotoxic activity of spleen cells from mice immunized with 5×10^6 Sp6/B7 cells reached the same levels found in mice injected three times s.c. with a 10-fold lower dose of tumour cells (Fig. 2, compare histogram 3 s.c. -0.5 with histogram 1 s.c. -5).

Immunization with 5×10^6 Sp6/B7 cells i.p. afforded much less protection against i.p. challenge with WT Sp6 (71% tumour incidence) than that elicited by s.c. immunization (20–50% tumour incidence) (Table 2). The difference was statistically significant ($P < 0.01$). The protection was even less efficient than that observed in mice injected three times i.p. with 0.5×10^6 Sp6/B7 cells (57% tumour incidence). As attempts to inject 5×10^6 cells i.v. resulted in embolism, as described previously,²⁵ we injected 2×10^6 WT Sp6 and Sp6/B7 i.s., considering this number of cells to be the putative average amount reaching the spleen after an i.v. injection of 5×10^6 cells. Injection of Sp6/B7 i.s. did not give rise to any tumour growth (0% tumour incidence, as shown in Table 2), and afforded no protection against a challenge with WT Sp6 (100% tumour incidence, as shown in Table 2).

Extension of protection against s.c. challenge with WT Sp6 in a different anatomical quarter requires further immunizations

Full protection against s.c. challenge of 0.5×10^6 WT Sp6 cells in a different anatomical quarter was obtained in mice treated either with three s.c. injections of 0.5×10^6 Sp6/B7 cells or with a single s.c. dose of 5×10^6 Sp6/B7 cells, followed by two i.p. injections of WT Sp6 (0.5×10^6 cells). In these mice, protection was complete, with a tumour incidence of 0% (0/10 animals in the first group and 0/12 in the second). The Sp6-specific cytotoxic activity of mesenteric lymph-node-derived cells was comparable to that found in the spleen (Figs 2 and 4, compare histogram 3 s.c. + 2 i.p. -0.5 with histogram 3 s.c. + 2 i.p.).

The Sp6-specific cytotoxic effector cell population mostly belongs to the CD8⁺ T-cell subset

Purification of spleen-derived lymphoid cell populations showed that most of the cytotoxic activity observed in Sp6/B7-Sp6-injected animals is attributable to the CD8⁺ T-cell subset (Fig. 5).

DISCUSSION

Tumour cells modified by B7 expression are known to become immunogenic and to present hampered growth *in vivo*.^{4–6} Accordingly, Sp6 cells become immunogenic when expressing *de novo* B7-1 and do not give rise to progressively growing tumours *in vivo*, regardless of the route of delivery, unless B7-1 cDNA is deleted. This implies that most, if not all, Sp6/B7 cells have been eliminated and only B7-1 loss variants can grow.^{8,22} In addition, impaired growth of B7-positive tumour cells does

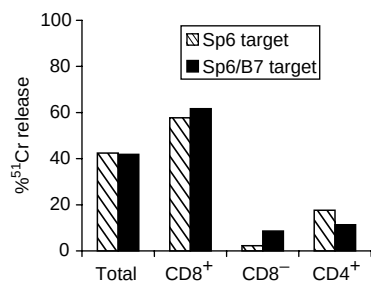


Figure 5. Cytotoxic response against wild-type (WT) Sp6 and Sp6/B7 of total and purified CD8⁺, CD8⁻ and CD4⁺ spleen cell populations of mice injected multiple times with 0.5×10^6 Sp6/B7-WT Sp6 cells s.c. and i.p., expressed as % ⁵¹Cr release. Purification of the different spleen cell populations was performed as indicated in the Materials and methods. Each histogram indicates the percentage lysis from the pool of cells from spleens of about five animals. Mean values and standard deviations in this experiment are therefore not given. Black histograms show the percentage lysis of Sp6/B7 targets, and striped histograms the percentage lysis of WT Sp6 targets. As indicated in the Materials and methods, all experiments were performed with a 100 : 1 effector/target ratio. The type of effector cell population from each group of histograms is indicated on the abscissa.

not depend on an intrinsic 'weakness' of Sp6/B7 compared with WT Sp6, as both cell types exhibit comparable replication rates *in vitro* (data not shown). The mechanism of Sp6/B7 elimination involves the immune system, and mostly the specific T-cell response, although not exclusively, as we have found that 85% (not 100%) of nude mice injected with Sp6/B7 cells developed tumours (data not shown). It has been reported that expression of B7-1 in tumour cells can decrease tumorigenicity by increasing NK-mediated tumour cell killing in normal and nude mice.^{7,9} In addition, nude mice may present greater NK cell activity than immunocompetent mice.⁹ Therefore, we hypothesize that 15% of nude mice rejecting Sp6/B7 may display greater NK activity than the majority of mice in their group, and are capable of inhibiting growth of B7-1⁺, but not B7-1⁻, Sp6 tumours.

In order, however, to induce an immune response resulting in rejection of WT Sp6, the three parameters of antigen dose, site of delivery and time elapsing between immunization and challenge, are crucial. Although these parameters are closely interlinked, their weight in the tumour model considered is very different. The anatomical site of delivery of Sp6/B7 plays the most important role, determining the degree of protection against WT Sp6. Only the s.c. route of delivery was able to provide appreciable protection against successive challenges with WT Sp6 and activate tumour-specific CTLs displaying the same levels of cytotoxicity against both Sp6/B7 and WT Sp6 (Fig. 2).

A single dose of 0.5×10^6 Sp6/B7 cells, given s.c., elicited a local immune response. The protective response against WT Sp6 could be extended to other anatomical locations by increasing the dose of Sp6/B7 cells delivered s.c., with either three repeated injections of the same dose (0.5×10^6 cells) or a 10-fold higher single dose (5×10^6 cells) (Tables 2–4). Analysis of the cytotoxic activity of cells from secondary lymphoid organs of differently injected mice suggests a scenario whereby

a single dose of 0.5×10^6 Sp6/B7 cells given s.c. elicited a local immune response at the draining lymph node. Some memory cells remained localized there, whilst others reached the spleen, where an expansion of tumour-specific memory T-cell clones started,²⁶ although to an extent insufficient to export WT tumour protection to other anatomical sites. Increasing the dose of antigen further raises the expansion of tumour-specific T-cell clones and their spread to secondary lymphoid organs, allowing anatomical extension of tumour protection. Thus, the expansion and spread of tumour-specific CTL precursors are functions of the tumour-antigen dose. Conversely, too large an amount of antigen may prove deleterious by inducing tolerance.^{14,16} In the tumour system presented here, we have, to date, not been able to determine the tolerogenic dose: WT Sp6 was rejected even after a total of 10 injections of Sp6/B7-Sp6 (data not shown).

The i.p. route afforded partial protection against WT tumour cells either with repeated injections of Sp6/B7 or, to a lesser extent, with a single bolus containing 10 times the number of cells delivered repeatedly (Tables 2 and 4). Accordingly, some CTL activity against WT Sp6 was found in spleen cells from mice injected i.p. with Sp6/B7, suggesting that some CTL memory response was triggered (Fig. 2). The fact that i.p. immunized mice are less protected against challenge with WT Sp6 could be caused by an increase in the tumour growth rate in that anatomical location, with consequent impairment of the CTL/tumour cell ratio. In naive mice, inocula of the same number of cells of WT Sp6 grew faster when given i.p. than when administered s.c. or i.v. (data not shown).

The i.v. route, with consequent involvement of the spleen, is historically considered as route for tolerance induction. The fact, however, that Sp6/B7 cells injected i.v. did not give rise to a protective immune response against WT Sp6 cannot be explained in terms of tolerance. Sp6/B7, injected i.v., showed limited growth (as did Sp6/B7 injected s.c. and i.p.) after the first injection (Table 1) and no growth at all after two further injections (Table 4). That is to say, an immune response was mounted against Sp6/B7 cells, but not against WT Sp6 cells, as also suggested by the cytotoxicity assays (Fig. 2). In this case, cytotoxicity would appear to be sustained by B7-1-dependent effector CTLs, rather than by memory CTLs. Similar events were observed after i.s. inoculation (Table 2 and data not shown), as also described by others in the TS/A mammary adenocarcinoma model.²⁰ This, however, is not always the case: in other tumour systems (e.g. MC-GP fibrosarcoma), the i.s. route has been shown to induce a protective immune response.²⁵

In conclusion, this work shows that in the Sp6 tumour model, the anatomical location of antigen delivery in its immunogenic form (i.e. Sp6/B7) would appear to play the most important role in determining the outcome of a protective immune response against the WT tumour. The antigen dose regulates the anatomical extent of this protection. These results are consistent with the current view of immune reactivity.^{14–16}

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