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Antitumor Immunity Elicited by Tumor Cells Transfected with B7–2, a Second Ligand for CD28/CTLA-4 Costimulatory Molecules

Guchen Yang, Karl Erik Hellström, Ingegerd Hellström, and Lieping Chen

Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA 98121

We have examined the role of the B7–2 costimulatory molecule, a second ligand for CD28/CTLA-4 counter-receptors, in the induction of antitumor immunity. A plasmid containing murine B7–2 cDNA was transfected into the immunogenic mouse mastocytoma P815 of DBA/2 origin. In contrast to the lethal growth of the wild-type (wt) P815 tumor, B7–2-positive (B7–2+) P815 cells inoculated into syngeneic mice regressed, and immunization of mice with such tumor cells protected them against the challenge of wt P815 tumor. Depletion of CD8+, but not of CD4+, lymphocytes in vivo by specific Abs abolished the regression of B7–2+ P815 tumors. CD8+ cytolytic T cells could be generated from mice immunized with B7–2+ P815. They were found to be MHC class I-restricted and specific for the P815 tumor. In contrast, transfection of the B7–2 gene into the nonimmunogenic MCAl02 fibrosarcoma of C57BL/6 origin induced neither tumor regression nor protective immunity. Co-expression on MCAl02 cells of B7–2 together with the related costimulator B7–1 also failed to induce immunity to MCAl02 tumor. Our results indicate that transfection of B7–2 into tumor cells can improve host response to some tumors, and that the effects seen are similar to those previously observed for B7–1.


Two combined signals provided by APCs are required for T cell activation. The first one is mediated via the TCR following its interaction with antigenic peptide-MHC complexes, and the second is delivered by accessory or costimulatory molecules through their counter-receptors on the T lymphocytes. In the absence of costimulation, exposure of T cells to an Ag may cause unresponsiveness or anergy (1–4).

Costimulation mediated by B7–1 (B7, CD80) plays an important role in the induction of T cell-mediated antitumor immunity (5). By transfection of the murine B7–1 gene into mouse tumor cells, protective and sometimes curative immunity against wild-type (wt) tumors has been induced in several mouse models (6–10), with the antitumor immunity being mediated by CD8+ (6, 7, 9), CD4+ (8) T cell populations, or both (10). Tumor immunogenicity appears to be an important factor in determining the effect of B7–1 costimulation, because all of four immunogenic tumors tested were rejected when inoculated into syngeneic mice following transduction of the B7–1 gene, as compared with none of four nonimmunogenic tumors (11).

Recently, a second ligand for CD28/CTLA-4, B7–2 or B70, has been identified (12–23), with both the human (13, 14) and the mouse (15, 16) genes isolated. The mouse and the human B7–2 molecules have approximately only 25% amino acid similarity with the respective B7–1 gene (13–16). Like B7–1, B7–2 is not expressed by the majority of carcinomas and sarcomas (11), and unlike the inducible expression of B7–1, B7–2 is expressed constitutively on monocytes and dendritic cells (14, 20). It also appears on the surface of B cells earlier than B7–1 after exposure to LPS, Con A, or cAMP (13, 20). The costimulatory function of B7–2 on the response of CD4+ cells has been examined, and it has been shown that murine B7–2 expressed on activated B cells or transfected COS cells can costimulate both T cell proliferation and IL-2 production (13, 17, 18, 22). Blocking of both B7–1 and B7–2 in vitro by specific mAbs was found to induce T cell anergy and to down-regulate the transcription of several lymphokines, including IL-2, IL-3, IL-4, and IFN-γ (24). A fusion protein, CTLA4Ig, that blocks the binding of B7–2 and B7–1 to CD28/CTLA-4, can greatly inhibit a primary allogeneic
mixed lymphocyte reaction in vitro mediated by lymphocytes from either normal or B7−1−deficient mice (14, 15, 20), and administration of an anti-B7−2 mAb can inhibit a T cell-dependent Ab response in vivo (18). Taken together, these results suggest that the B7−2 molecule is another potent costimulator for the initiation of a T cell immune response to tumor Ags.

To investigate the role of costimulation of antitumor immunity by B7−2, we transfected the murine B7−2 gene into the P815 mastocytoma and the MCA102 fibrosarcoma that originated in DBA/2 and C57BL/6 mice, respectively. The P815 mastocytoma is immunogenic, according to standard immunization and challenge experiments (11), and it expresses a well characterized tumor-associated Ag, P1A (25), that is a target for CD8+ CTLs. In contrast, MCA102 is nonimmunogenic when tested by the same methods (11, 26). We report in this work that transfection of B7−2 can vastly increase the in vivo immunogenicity of P815 tumor cells and amplify a CD8+ CTL response, but transfection of the B7−2 gene does not make the MCA102 tumor immunogenic.

Materials and Methods

Mice

Female DBA/2 (D2), BALB/c (nu/nu), and C57BL/6 (B6) mice, 4- to 6-wk-old, were purchased from The Jackson Laboratory (Bar Harbor, ME).

Cell lines

The D2-derived mastocytoma P815, T lymphoma L1210 and L178Y and the B6-derived thymoma EL-4 were purchased from American Type Culture Collection (ATCC, Rockville, MD). The B6-derived fibrosarcoma MCA102 was provided by Dr. J. Mult (then at the National Cancer Institute, Bethesda, MD). Both P815 and MCA102 are negative for the expression of B7−1 and B7−2, according to FACS analysis (11). The B7−1+ P815 and B7−1+ MCA102 clones have been generated previously in our laboratory by infection of P815 and MCA102 with pLXSN or pLNSK recombinant retroviruses containing murine B7−1 cDNA (11). The mock P815 and mock MCA102 clones were derived similarly, but infected with parental retroviruses (11). YAC-1 is an NK-sensitive lymphoma (ATCC). All cells were maintained at 37°C in DMEM containing 10% FCS (referred to as “medium”).

Cloning and transfection of murine B7−2

A DNA fragment encoding the entire open reading frame of murine B7−2 was amplified by reverse transcription-coupled PCR (PCR) from RNA prepared from LPS-activated murine spleen cells (15). The sense primer (5'-TCGATAGGCAATCTCTACTATTCGGTTACAGACTCT-3') consists of an oligonucleotide corresponding to 64 to 83 nucleotides of murine B7−2 cDNA, plus a restriction site for EcoRI. The antisense primer (5'-TACGATACCGGAGTACTTCTACTGAGTCTACACTGTAGC-3') corresponds to 1018 to 1039 nucleotides of murine B7−2 cDNA, plus a site for XhoI. The PCR product was cloned directly into the vector pLXSHD (provided by Dr. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA), and the structure of the resulting construct pLXSHDmB7−2 is shown in Figure 1A. The sequence of murine B7−2 was verified by DNA-sequencing analysis. The vector was transfected by electroporation into wt P815, wt MCA102, and B7−1+ MCA102 tumor lines. The transfectants were selected in medium containing 5 mM histidinol D (Sigma Chemical Co., St. Louis, MO). Individual histidinol D-resistant colonies were picked and stained by GL1 mAb (see the following).

FACS analysis

The transfectants were incubated for 30 min at 4°C with FITC-conjugated anti-B7−2 mAb GL1 (18) or anti-B7−1 mAb 1G10 (28) (PharMingen, San Diego, CA) at 10 μg/ml in medium (6). Transfectants were also incubated with CTLA-4 Ig (6), which binds to both B7−1 and B7−2, washed, and incubated for an additional 30 min at 4°C with FITC-conjugated goat anti-human Ig G4 serum (Tago Inc., Burlingame, CA). Stained cells were analyzed on an EPICS C flow cytometer. The expression of MHC class I molecules was detected by the same procedure by using mAbs for K b (SF-1.1.1), D b (34–4–21S), or L d (28–14–8S). These mAbs were purified from the culture supernatants of hybridomas (ATCC) by affinity chromatography, with protein A coupled to Sepharose CL-4B (Pharmacia Fin Chemicals, Piscataway, NJ).

Generation of CTLs

Methods for induction of CTLs, and for assaying their activity on tumor cells, have been described (29). Briefly, mice were injected s.c. with mock, B7−1+, or B7−2+ P815 cells, and the resulting tumor nodules were removed surgically on day 10. Two weeks after tumor removal, spleen cells were prepared and cocultivated with γ-irradiated (12,000 rad) wt P815 cells in 24-well plates (Costar, Cambridge, MA) for 5 days. The cytolytic activity of bulk-cultured spleen cells was examined in a standard 4-h 51Cr release assay with different E:T ratios, as indicated in the figure legends. For in vitro T cell-depletion experiments, effector cells at 1 × 106 per ml were pretreated for 60 min at 4°C with 20 μg/ml mAb, as indicated, together with 1/32 diluted rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada), and washed twice before use.

Animal studies

Tumor cells at 1 × 106/ml (P815-derived cells) or 2 × 106/ml (MCA102-derived cells) were injected into the shaved right back of syngeneic, and in some experiments, athymic mice in a 0.1-ml volume. The mice were scored for tumor growth once per week, and tumor size was documented by measuring two perpendicular diameters in millimeters by using a caliper. For experiments conducted to deplete CD4+ or CD8+ T cell populations in vivo, purified mAbs to CD4 (GK1.5) or CD8 (116-13.1) were injected i.p. 1 day before tumor inoculation at 1 mg per mouse. The same amount of mAb 10.2 (anti-human CD5) was given as a control. For analysis of efficiency of the depletion, single cell suspensions were prepared from spleens 4 to 5 wk after mAb treatment, incubated with FITC-conjugated anti-CD4 (L3T4) or anti-CD8 (Lyt-2) mAbs (PharMingen), and examined by FACS analysis. For immunization experiments, mice were injected into the shaved right back with live tumor cells by using the same procedure as described above, and tumor nodules were removed surgically at day 10. Two weeks after tumor removal, the mice were challenged into the left back or the flank with wt tumor cells at 1 × 106/ml (P815) or 2 × 106/ml (MCA102). The experiments were terminated when the tumors reached 20 to 25 mm in diameter, severe ulceration and bleeding had developed, or the mice had died. The results are expressed as mean diameter (in millimeters) of tumors from groups of five to ten mice each.

Results

Expression of the murine B7−2 gene in P815 and MCA102 tumor lines

The pLXSHDmB7−2 plasmid containing murine B7−2 cDNA (Fig. 1A) was transfected into mastocytoma P815 and fibrosarcoma MCA102. The transfectants were selected for histidinol D resistance and tested for expression of B7−2 by flow cytometry after immunofluorescent staining with either a specific mAb to B7−2 (GL1) or the soluble ligand CTLA-4 Ig that binds to both B7−1 and B7−2. Clones that stably expressed B7−2 were selected for further analysis. As shown in Fig. 1B, a transfectant from P815, B7−2+ P815, and a transfectant from MCA102, B7−2+ MCA102, were stained by GL1 and CTLA-4 Ig.
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A. EcoRI XhoI pLXSHDmB7-2

B. Anti-67.1 (1G10) Anti-67.2 (GL1)

C: Expression of MHC class I Ags on P815 transfectants. The mock, B7-2+, or B7-1+ P815 tumor cells were stained with FITC-conjugated goat anti-mouse Ig Cy serum. A total of 5000 cells was analyzed for each sample.

but not by mAb 1G10, which recognizes B7-1. On the other hand, the B7-1+ P815 (a B7-1-transfected P815 clone) and the B7-1+ MCA102 (a B7-1-transfected MCA102 clone) were not stained by GL1, but stained with 1G10 or CTLA4 Ig. Neither B7-1 nor B7-2 was detectable in mock-transfected P815 (mock.P815) or mock-transfected MCA102 (mock.MCA102) cells. B7-1+ MCA102 cells were also transfected with the pLXSHDmB7-2 plasmid, and a clone expressing both B7-1 and B7-2 (B7-1+/B7-2+ MCA102) was selected (Fig. 1B) for additional experiments.

The expression of MHC class I molecules on B7-2 transfectants from P815 was also examined by using specific mAb to H-2Kd, Dd, and Ld. The B7-2+ P815 cells, as well as B7-1+ P815 and mock.P815 cells, expressed similar levels of all three MHC class I Ags (Fig. 1C). B7-2 transfectants derived from MCA102 did not express detectable levels of MHC class I Ags (data not shown).

Tumorigenicity following transfection of the B7-2 gene

To examine their tumorigenicity, B7-2+ P815 and B7-2+ MCA102 cells were injected s.c. into syngeneic D2 or B6 mice, respectively; B7-1+ and mock-transfected cells from the respective tumors were injected as controls. The mock-transfected tumors grew progressively and killed the mice. In contrast, B7-2+ P815 cells, containing either the B7-2 or B7-1 genes, completely regressed after a transient growth (Fig. 2A). Similarly to what was previously reported for B7-1+ P815 (11), tumors induced by B7-2+ P815 cells grew progressively in athymic mice (Fig. 2B), suggesting that T cells were responsible for their rejection in the immunocompetent mice.

In contrast to the vastly increased immunogenicity of B7-2+ P815 cells, B7-2+ MCA102 tumor grew as well as mock.MCA102 cells in immunocompetent mice (Fig. 3A). Similar results were obtained by using B7-1+/B7-2+...
MCA102 cells that express both B7-1 and B7-2 at high levels (Fig. 3B). Therefore, as reported previously for B7-1 (11), transfection of B7-2 into MCA102 tumor does not make it immunogenic, not even when the two costimulatory molecules are co-expressed.

Role of CD4+ and CD8+ lymphocytes in the rejection of B7-2+ P815 tumors

To examine the role of CD4+ and CD8+ T cell subsets in the rejection of the B7-2+ P815 tumor, mice were injected with mAbs to deplete either subset before the inoculation of B7-2+ P815 cells. This procedure removed the majority of respective population of T cells without detectably affecting the other cell population, as judged by flow cytometry on the day in which experiments were terminated. In a representative experiment, the spleen cells from the mice treated with anti-CD4 mAb retained 95% of CD8+ and <5% of CD4+ T cells, and those treated with anti-CD8 mAb had 7% of CD8+ and 100% of CD4+ T cells, as compared with the mice treated with the control anti-human CD5 mAb. The removal of CD8+ T cells completely abrogated the rejection of this tumor, whereas injection of either an anti-CD4 or a control mAb had no detectable effect (Fig. 4). These results demonstrate that rejection of B7-2+ P815 was mediated primarily by CD4+ CD8+ T cells.

The role of B7-2 costimulation in the induction of CTL activity was also studied. Bulk-cultured CTL was generated, as described in Materials and Methods, from mice immunized by injection of P815 cells that had been mock-transduced or contained either the B7-1+ or the B7-2+ gene, and their activity was examined by using wt P815 cells as the target. CTL activity of spleen cells from mice immunized by either B7-2+ P815 or B7-1+ P815 was significantly higher than that of spleen cells from mice immunized by mock-P815 (Fig. 5A). The CTLs from B7-2+ P815-immunized mice were specific for P815 cells in that they did not lyse syngeneic L1210 and L5178Y lymphoma cells, allogeneic EL-4 lymphoma cells, nor NK-sensitive YAC-1 cells (Fig. 5B). The cytolytic activity was abolished completely by treatment of the effector cells in the presence of complement with an anti-CD8 mAb, whereas an anti-CD4 mAb had no effect (Fig. 5C). We also examined whether expression of B7-2 or B7-1 by the target cells influenced their sensitivity to lysis by CTLs. No significant differences were observed by using wt, B7-2+ or B7-1+ P815 cells as the target cells and CTLs from either B7-2+ P815- (Fig. 6A) or B7-1+ P815-immunized mice (Fig. 6B). We conclude that costimulation of tumor immunity by B7-2 operates at least partially via the induction of a tumor-specific CD8+ CTL response.

Effect of B7-2-transfectants on the induction of systemic immunity against wild type tumors

The ability of both B7-2+ P815 and B7-2+ MCA102 cells to induce systemic immunity against the respective wt tumors was examined. Mice were immunized by a single s.c. injection of live tumor cells, followed by surgical removal of the tumor nodules. Two weeks later, they were challenged with a lethal dose of wt cells. Immunization with mock-P815 showed only a moderate protective effect, whereas immunization by B7-2+ P815 cells completely protected the mice from tumor challenge (Table I). This protective effect was similar to that induced by immunization with B7-1+ P815 cells. No such protective effect was seen in the MCA102 tumor system (Table I).
Discussion

We have demonstrated that the costimulatory molecule B7–2 expressed on transfected P815 tumor cells can amplify both local and systemic antitumor immunity. This effect appears to result primarily from an enhanced generation of CD8+ T cells with a cytolytic activity specific for this tumor. These findings extend the previous observation that immunization with tumor cells transfected to express B7–1, the first discovered ligand for CD28/CTLA-4, can enhance the induction of CD8+ CTLs and vastly increase tumor rejection response, also against wt tumors (6, 10, 11).

When comparing the effects of transfection of the B7–2 or the B7–1 genes into P815, essentially the same results were observed, with tumors expressing either gene regressing in immunocompetent hosts after a transient growth (Fig. 2). Furthermore, CTL activity induced by immunization with either B7–2+ or B7–1+ P815 cells was comparable, although bulk-cultured CTLs from mice immunized with B7–1+ P815 had slightly higher CTL activity than that seen with CTLs generated from mice immunized with B7–2+ P815 in several parallel comparison experiments (Fig. 5). FACS analysis of the B7–1 and B7–2 expression by P815 transfectants using CTLA4Ig.
which binds both B7-1 and B7-2, indicates that B7-1 was expressed at a higher level than B7-2 on those transfectants. Therefore, it is difficult to quantitatively compare the level of antitumor immunity induced by B7-2+ and B7-1+ P815 cells. Nevertheless, our results suggest that there are no qualitative differences between the ability of these two transfectants to induce antitumor immunity.

The dominant function of B7-2 costimulation in the P815 tumor model appears to be facilitation of the generation of a CD8+ T cell response with CTL activity in vitro (Figs. 4 and 5). Our data suggest that the amplification of P815-reactive CD8+ CTLs in vivo does not require help from CD4+ T cells, which is similar to observations made with B7-1+ P815 cells (Yang et al., unpublished data). In contrast, transfection of B7-1 into murine melanoma cells (10), which expresses the membrane-associated tumor Ag p97 (30), or into Sal sarcoma transfected with a truncated MHC class II gene (8), can costimulate a CD4+ T cell-mediated response against the respective tumors. Neither wt P815 nor its B7 transfectants express MHC class II molecules (data not shown); this lack and/or failure of tumor Ags to be released from P815 cells, followed by processing in professional APCs and presentation by their MHC class II molecules may explain the lack of a CD4+ T cell response against this tumor.

Several recent studies indicate that the presence of B7-1 and B7-like molecules can increase the sensitivity of tumor cells to lysis by CTLs. Expression of B7/BB1 by a human ovarian carcinoma line increased its sensitivity to lysis by anti-CD3 redirected CTLs (31); transfection of the murine J558 plasmacytoma with B7-1 cDNA enhanced its sensitivity to CTL lysis, and this was significantly blocked by an anti-B7-1 mAb (9). However, in other studies, B7 transfection had no such effect (32–34). Our results agree with the latter set of findings, as neither transfection of B7-2, nor of B7-1, enhanced the effector function of CTLs on P815 cells (Fig. 6).

Transfection of B7-2 into MCA102 failed to make it immunogenic (Fig. 3A; Table I), even when B7-2 was co-expressed with B7-1 (Fig. 3B), and lack of costimulation is, therefore, not the reason that MCA102 is nonimmunogenic. A defect in Ag processing, presentation and/or a lack of tumor antigen(s) is a more likely explanation. Failure to express MHC class I molecules (35) and down-regulation of transporters for Ag processing (36, 37) are relatively frequent in tumors. Because MCA102 cells express undetectable levels of MHC class I Ags (11, 26), and as we have recently found that co-expression of B7-1 (B7-2 not yet tested) with MHC class I Ags can make it immunogenic (Yang et al., unpublished data), the lack of immunogenicity of MCA102 is probably caused by its lack of MHC class I molecules. Although our data indicate that transfection of B7-2 has an effect similar to that of transfection of B7-1 on the induction of tumor immunity, further work is needed to delineate differences (if they exist) with the ultimate goal to elicit a therapeutically useful antitumor immune response.

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References

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