Cancer Immunol Immunother (1994) 39: 342–345

Cancer Immunology Immunotherapy

Short communication

Recruitment of helper T cells for induction of tumour rejection by cytolytic T lymphocytes

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Received: 13 July 1994/Accepted: 3 August 1994

Abstract. Immunotherapy of cancer could be possible in cases in which competent effector T cells can be induced. Such an approach depends on expression of tumour-specific antigens by the tumour cells and on the availability of sufficient costimulatory support for activation of cytotoxic T lymphocytes. Here, a strategy for helper T cell recruitment for induction of tumour-specific cytotoxic immune responses is presented. Allogenic MHC class II molecules were introduced into tumour cells by cell fusion. These hybrid cells, when injected into mice, induced rejection of an established tumour. The contribution of CD4-expressing helper T cells in the induction phase and of CD8-expressing T cells in the effector phase of the immune response was demonstrated. The approach described could be applicable to cases in which a suitable tumour antigen is present but not identified; it employs regulatory interactions that govern physiological immune responses and is designed to be minimally invasive.

Key words: Tumour rejection – CTL – Helper cells

Introduction

Cancer, besides having specific causes, indicates a failure in immune surveillance [1]. An increasing number of tumour antigens are being demonstrated [2, 3] and, in several cases, tumour-specific cytotoxic T lymphocytes (CTL) have been isolated [4–7]. However, these cells are often not effective against the tumour. This incompetence of tumour-specific CTL can be seen, in part, as a consequence of a lack of costimulatory support during their activation. Consequently attempts are being made to provide costimulating factors like lymphokines directly [8] or to introduce genes into the tumour cells that code for lymphokines [9, 10] or for B7 [11, 12], an adhesion molecule on B cells that is the ligand of CD28, a coactivating receptor on T cells. Since, under physiological conditions, helper T cells are the source of coactivating factors, we tested strategies for helper T cell recruitment to support the induction of tumour-specific CTL.

In a previously reported study [13] we investigated the conditions and the cellular requirements for productive collaboration of helper and cytotoxic T cells that lead to effective cytotoxic responses. Using a well-defined in vitro system, we could show that both cells have to come together on the surface of antigen-presenting cells that present antigens on MHC class I molecules for recognition by CTL as well as on MHC class II molecules for stimulation of helper T cells. The specificities of the two cells are not relevant for successful collaboration. Direct antigen-dependent, T-cell-receptor-driven interaction is not required. Their contact is mediated by the antigen-presenting cell. We hypothesized that the mechanisms observed in vitro could also govern helper-dependent primary induction of CTL in vivo. Transfer of MHC class II molecules to a tumour could lead to induction of cytolytic effector cells and eradication of the tumour via helper T cell recruitment and activation. We tested this strategy in a mouse tumour model.

Materials and methods

Animals, cells and antibodies. C57Bl/6 mice were bred in the animal facilities of the Max-Planck-Institut für Biologie and used at an age of 6–10 weeks. Tumour inoculation experiments were terminated after 25 days to avoid unnecessary suffering of the animals.

The tumour cell lines EL4 (H-2^b:K^b, D^b) [14], RMA (H-2^b:K^b, D^b) [15] and A20 (H-2^d:K^d, A^d, E^d, D^d, L^d) [16] were cultured in Dulbecco's minimal essential medium (Gibco, Eggenstein, Germany) supplemented with heat-inactivated fetal calf serum (5%), glutamine (2 mM), *N*-2-hydroxyethylpiperazine-2-ethanesulphonic acid (5 mM), mercaptoethanol (μM), penicillin (100 U/ml) and streptomycin (100 μg/ml).

The monoclonal antibodies used in these studies (B8-24-3, anti-H2-K^b, ATCC, Rockville, Md., USA; 25.9.17, anti-A^d, A^b; K24.199.1,

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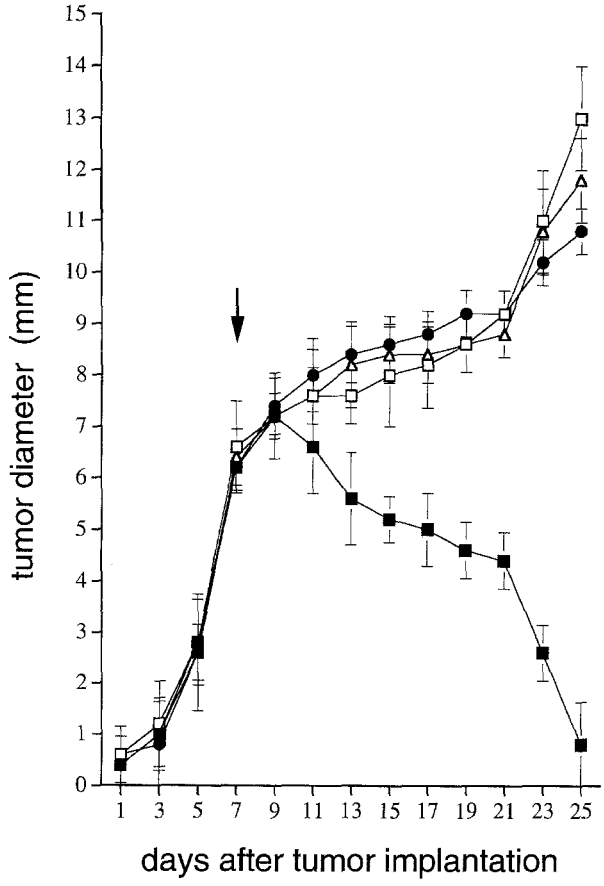

Fig. 1. Rejection of established tumours. Samples containing 1×10^6 viable EL4 tumour cells (H-2b-class-I-positive, class-II negative) were implanted intracutaneously into the backs of syngeneic C57Bl/6 mice; 7 days later (*arrow*), four groups of mice (five mice per group) were injected intraperitoneally with 5×10^6 EL4-A20 hybrid cells (■), with a mixture of 5×10^6 EL4 and 5×10^6 A20 (□), with 5×10^6 EL4 alone (Δ) or with phosphate-buffered saline (PBS; ●). All cells used for immunization were irradiated (33 Gy). EL4 and A20 cells that had been injected separately were subjected to the same electrofusion protocol as EL4-A20 hybrid cells. Tumour growth was measured using a vernier caliper

anti-A^d; T1.31C, anti-H2-K^d/D^d; GK1.5, anti-CD4; 19.178, anti-CD8) were affinity-purified on protein-A- or protein-G-Sepharose (Pharmacia, Freiburg, Germany).

Hybrid cell formation by electrofusion. Fusion of cells was performed using a modified electric-field-induced fusion protocol [17]. Briefly, 10×10^6 cells were suspended in 0.5 ml sucrose (0.3 M), dielectrophoretically aligned in an inhomogeneous electric field (150 V/cm, 10 s) and fused with a pulse of 500 V/cm, 25 μF using a BioRad gene pulser. Fusion efficiency in the case of EL4-A20 and RMA-A20 fusions was 30%–40% when counted by microscopy and more than 90% when analysed by two-colour fluorescence flow cytometry, using antibodies directed against H-2K^b (B8-24-3) and H-2A^d (25.9.17). Different cell types required some adjustment of the fusion conditions, especially, of voltage and time for the alignment and voltage and capacity used for induction of fusion.

Results and discussion

EL4 cells, which are thymoma cells of C57Bl/6 origin and express MHC class I proteins of the b haplotype, were implanted intracutaneously in the backs of syngenic C57Bl/6 mice. The cells grew as solid tumour which, after 7 days, reached a size of 6–8 mm in diameter. At this time the animals were injected intraperitoneally with EL4 cells that had been fused by electrofusion with A20 cells immediately before injection. A20 is a B lymphoma cell type that expresses MHC class I and class II molecules of the H-2^d haplotype. Thus, these hybrid cells carried tumour antigens of EL4 cells as well as allogenic MHC class I and class II molecules, and should be potent stimulators of a high-frequency response of allospecific $CD4^+$ helper T cells. The inoculum was irradiated to prevent propagation of the lymphoma cells. Regression of the tumour was observed 3 days after injection, and by day 25 it was completely eradicated (Fig. 1). Cured animals did not develop any sign of tumour growth again and were immune to challenges with EL4 cells for more than 3 months. The tumour continued to grow progressively, however, in mice that had been injected with a mixture of EL4 and A20 or with EL4 alone or with phosphate-buffered saline instead of hybrid cells (Fig. 1). The anti-tumour response described here was specific for EL4, since mice that had been immunized with EL4-A20 hybrid cells did not reject a different thymoma cell line, RMA (data not shown). These results clearly show rejection of an established tumour and could provide a basis for developing a new strategy for immunotherapy of cancers. As described for helper-T-cell-dependent induction of CTL in vitro [13], in the in vivo model presented here, the antigens for both T cell types also have to be present on one cell in order to be effective for induction of cytolytic activities. A mixture of tumour and MHC-class-II-expressing cells was, obviously, not sufficient for coupling regulatory and effector stages of the immune response against the tumour.

The same requirements were found for immunizations against tumours. Mice that had been injected with hybrid cells of EL4 and A20 became immune to the tumour and rejected EL4 cells that were implanted intracutaneously in the backs of the animals 3 weeks after immunization (Fig. 2A). As before, mice that had been injected with a mixture of EL4 and A20 or with any one of the two cell types separately were not protected (Fig. 2A). Mice that had been immunized with RMA-A20 hybrid cells rejected RMA tumours (four out of five animals) but not EL4 tumours (none of five animals). Conversely, none of five mice that had been immunized with EL4-A20 hybrid cells rejected RMA tumours. Development of immunity was prevented in animals that, together with the hybrid cells used for immunization, received monoclonal antibodies with specificity for the coreceptor of helper T cells, CD4, for MHC class II molecules of the H-2^d haplotype, which are borne by the allogenic fusion partner of EL4, and for CD8, the coreceptor of CTL (Fig. 2B). A monoclonal antibody that is specific for the MHC class I allomorphs of A20 (H-$2K^d$ and H-$2D^d$) had no effect. Thus, $CD4^+$ helper T cells as well as $CD8^+$ CTL of the C57Bl/6 recipient mice were involved in and essential for induction of tumour-specific immunity. At the effector stage, however, only antibodies specific for CD8 inhibited tumour rejection. Antibodies that would block helper T cell activation, anti-CD4 and anti-MHC class II, had no effect. These results

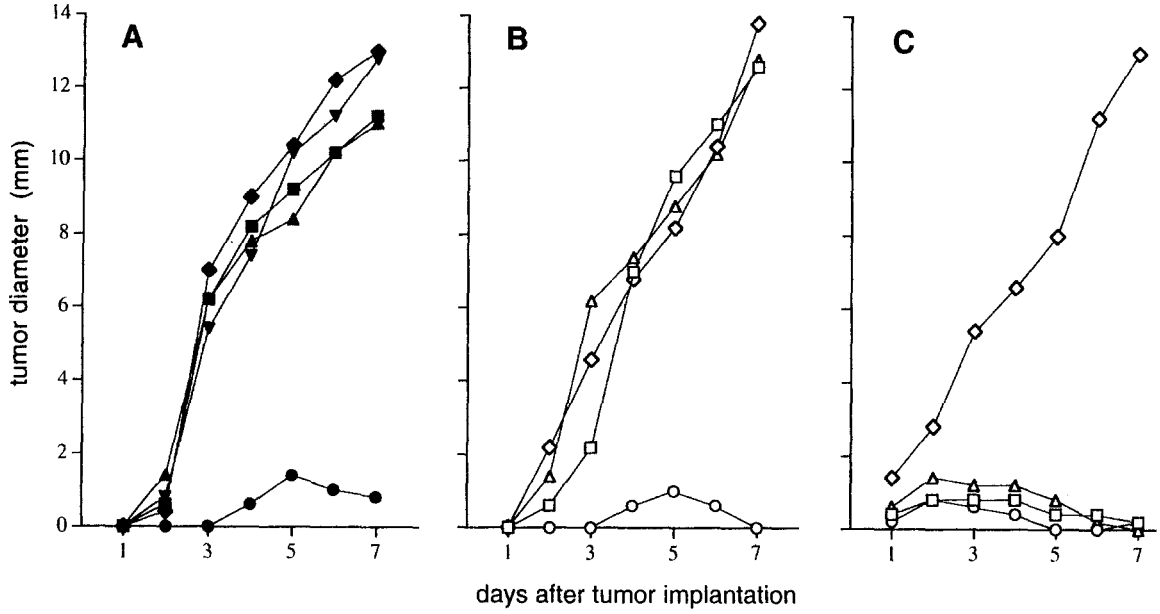

Fig. 2A–C. Induction of protective immunity to tumours. **A** Induction of antitumour immunity. C57Bl/6 mice (five mice per group) were immunized intraperitoneally with 5×10^6 EL4-A20 hybrid cells (●), 5×10^6 EL4 (◆), 5×10^6 A20 (▲), a mixture of 5×10^6 EL4 and 5×10^6 A20 (■) or PBS (▼). All cells were irradiated (33 Gy) prior to injection. Three weeks after immunization, 3×10^6 viable EL4 tumour cells were implanted intracutaneously into the backs of the mice. Tumour growth was monitored daily. **B** Effects of antibodies on the induction of immunity to EL4. Mice (five C57Bl/6 per group) were immunized with irradiated EL4-A20 hybrid cells as described in **A**. Antibodies directed against CD4 (GK1.5) (□), CD8 (19.178) (◇), A^d (K24.199.1) (Δ) and K^d/D^d (T1.31C) (○) were injected together with the hybrid cells. After 3 weeks, 3×10^6 viable EL4 cells were implanted intracutaneously into the backs of the mice. 0.5 mg of each antibody was injected per mouse. **C** Effects of antibodies on tumour rejection. Mice were immunized with irradiated EL4-A20 hybrid cells as described in **A**. After 3 weeks, antibodies were injected intraperitoneally and, at the same time, 3×10^6 viable tumour cells were implanted intracutaneously into the backs of the mice. The antibodies were anti-CD4 (□), anti-CD8 (◇), anti-A^d (Δ) and anti-K^d/D^d (○) as described before

demonstrate that protection against the tumour is dependent on $CD8^+$ CTL. $CD4^+$ helper T cells are required for induction of CTL but not for tumour rejection. Fusion of B lymphomas with the thymoma cells could also introduce new adhesion molecules into their membrane. The involvement of such adhesion receptors in the induction of the CTL activities has not yet been investigated. The results underscore the importance of helper T cells for the induction of CTL [18]. They also support our assumptions about the cellular requirements for collaboration of helper and cytotoxic T cells and the need for antigen linkage on the stimulator cell. It is conceivable that, in case of very extended tumours, activated CTL are rendered anergic by the tumour. Whether this indeed happens and whether such an anergic state could be overcome by repeated injections of hybrid cells needs to be analysed.

The strategy described here could provide the basis for immunotherapy of cancer. It would be applicable to cases in which specific tumour antigens exist that can be presented to MHC-class-I restricted CTL. Identification of the tumour antigens is not necessary. Genetic manipulation of the tumour cells by transfer of genes that code for single costimulatory factors for T cell activation is avoided, as is injection of recombinant cytokines, a treatment that is hampered by various side-effects. The approach in our experiments was designed to be minimally invasive. Only a sample with tumour cells is needed; cell fusion by an electrofusion protocol avoids chemical compounds and thereby extra toxicity controls; irradiation of the inoculum prior to injection prevents spreading of the tumor. A recent report on similar experiments in a rat model indicates that this approach can be applied to very different tumour types [19]. However, it still remains to be seen how far these observations can be generalized. The optimal treatment will depend on tumour type and medical indication so that different strategies, including those mentioned before, need to be developed and tested.

Acknowledgements. The general support of J. Klein, which made these experiments possible, is acknowledged. The animal experiments reported here were performed in agreement with the current German law on the Protection of Animals and have been approved by the responsible state authorities.

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