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Advances in Brief

Trioma-based Vaccination against B-Cell Lymphoma Confers Long-Lasting Tumor Immunity

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Abstract

A major goal of tumor immunotherapy is the induction of a systemic immune response against tumor antigens such as the tumor-specific immunoglobulin idiotype (Id) expressed by lymphomas of the B-cell lineage. We describe an approach based on specific redirection of the tumor Id toward professional antigen-presenting cells (APCs), thereby overcoming the inefficient presentation on the parental transformed B cell. Lymphoma cells are fused to a xenogeneic hybridoma cell line that secretes an antibody against a surface molecule on APCs. Due to preferential assembly between heavy and light chains of antibodies of different species-origin, the resulting "trioma" cells produce at high yield a bispecific antibody containing the lymphoma Id and the APC-binding arm, which redirects the Id to APCs. Processing and presentation of the Id will lead to T-cell activation. An absolute requirement for inducing a complete tumor protection was the immunization with antibody-secreting trioma cells as a cell-based vaccine instead of the soluble bispecific antibody. Tumor immunity was specific and long-lasting. Both $CD4^+$ and $CD8^+$ T cells were necessary for inducing tumor immunity.

Introduction

Activation of T cells requires two signals delivered by the APC.[2] The first signal is mediated by the antigen-MHC complex interacting with the T-cell receptor; the second signal is provided by costimulatory molecules expressed by the APCs, such as interleukin 2 or B7 that binds to CD28 on the T cell (1, 2). Dendritic cells and macrophages are potent professional APCs in that they are capable of providing costimulatory signals (2).

Despite the existence of TAAs and the presence of tumor-specific T cells in many experimental and human malignancies, cancer cells can escape the immune attack by several mechanisms. Defective signaling to T cells may in part result from inefficient presentation of TAAs by the tumor cell, *e.g.*, by down-regulation of MHC molecules (3) or alteration of antigen-processing pathways (4) or from the lack of costimulatory signals (5), which may even specifically anergize T cells (6). A major goal in tumor immunotherapy is to mount a systemic cytotoxic T-cell response of the tumor-bearing host against TAAs. Because in most cases, target antigens have not been molecularly identified, vaccination protocols have been developed using whole autologous tumor cells genetically modified to express cytokines or costimulatory surface molecules (7). The Ig Id expressed by lymphomas of the B-cell lineage constitutes a molecularly defined, tumor-specific antigen. Despite being able to present antigens to T cells (8, 9), B-cell malignancies can also evade the immune system, *e.g.*, because of insufficient delivery of costimulatory signals (10, 11). The Id of B-cell lymphomas has been exploited as a target in several active immunization protocols (12–17). Because of the low immunogenicity of the Id, it was necessary to administer the vaccine, which has been isolated from the tumor at the protein or the genetic level, together with adjuvants (12–16) or after coupling to granulocyte macrophage colony-stimulating factor (17).

Here we describe an approach that is based on specific redirection of the tumor Id toward professional APCs, thereby overcoming the inefficient presentation by the parental transformed B cell. Lymphoma cells are fused with a hybridoma expressing an Ab against an APC surface molecule. Due to preferential heavy/light chain pairing of species-different mAb (18), the hybrid cells produce at high yield a bsAb consisting of the lymphoma Id and the anti-APC specificity, which will initiate the uptake, processing, and presentation of the Id. The vaccinating effect of injecting the bsAb-secreting trioma cells turned out to be highly superior to that of the purified soluble bsAb.

Materials and Methods

Cell Culture Conditions and Generation of Trioma Cells. Murine A20 (ATCC TIB-208), murine MPC11 (ATCC CCL-167), and rat 2.4G2 (ATCC HB-197) cells were cultured in RPMI 1640 supplemented with 10% FCS and 50 μM 2-mercaptoethanol at 37°C in a humidified 5% CO_2 atmosphere. HAT-sensitive variants of the hybridoma were selected by culturing in 8-azaguanine. Five $\times 10^6$ cells of the fusion partner A20 were incubated in 2.5–5 mM iodoacetamide for 30 min at 4°C, washed with PBS, and mixed with 1.5×10^7 HAT-sensitive 2.4G2 cells. Fusion was done by treating with polyethylene glycol 1500 for 2 min. Cells were plated in 96-well dishes and exposed to HAT after 2–3 days.

Characterization and Purification of bsAb. Binding of the bsAb to Fcγ receptors and the presence of the A20-derived mouse Ig chains were examined by FACS. bsAb was coated on S49 (Thy-1^-) cells (ATCC TIB-36) and detected with FITC-labeled rat antimouse IgH or Igκ polyclonal Ab. The BiVneg variant lacked FcγR binding activity but contained the A20 Id and rat IgG, as was shown in the ELISA. For purification, bsAb from culture supernatants was loaded on an EconoPac protein A column (Bio-Rad Laboratories, Hercules, CA) and eluted with 0.1 M citric acid (pH 5.1) followed by cation exchange chromatography on Mono S (Pharmacia, Uppsala, Sweden).

Animal Studies. Groups of six female BALB/c mice (Charles River Breeding Laboratories) were injected i.p. with the immunogens in PBS, as indicated in Fig. 2. A boost was given after a 3-week interval. After another 7 days, the mice were challenged with 10^5 A20 tumor cells injected i.p. In some experiments, the immunizing cells were irradiated at a dose of 15 Gy from a Cs137 source. In the T-cell depletion experiments, the mice were injected i.p. with 200–500 μg of the anti-CD4 mAb GK1.5 (19) or the anti-CD8 mAb RmCD8 (20). The depletion was at least 99%, as was shown by FACS analysis. Depletion before immunization was done four times beginning 5 days before the first BiV delivery and discontinuing 7 days before the BiV boost (see Fig. 4*A*). At the time of tumor challenge, T cells had recovered. For depletion before tumor challenge, the depleting antibodies were delivered four times every 7–10 days starting 3 days before tumor inoculation (Fig. 4*B*). All experiments were done at least in duplicate. Statistical survival analysis was done using the log-rank test.

Anti-idiotypic ELISA. Tail vein blood was taken before and after immunization with BiV cells. ELISA plates were coated with A20 IgG2a purified from culture supernatants, incubated with serially diluted preimmune or im-

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[2] The abbreviations used are: APC, antigen-presenting cell; Ab, antibody; bsAb, bispecific Ab; Id, idiotype; Ig, immunoglobulin; TAA, tumor-associated antigen; HAT, hypoxanthine-aminopterin-thymidine medium; FACS, fluorescence-activated cell sorting; FPLC, fast protein liquid chromatography.

mune sera, and developed with peroxidase-labeled goat antimouse IgGFc, which had been adsorbed against mouse IgG2a. Reactivity of the sera with the constant domains of the A20 protein was excluded by a similar ELISA using an irrelevant IgG2a Ab as the capturing Ab. Humoral responses against other TAAs on the A20 cells were measured in a cellular ELISA. A20 cells were incubated with mouse sera and, subsequently, with peroxidase-conjugated goat antimouse IgGFc adsorbed against mouse IgG2a.

Results and Discussion

Conversion of a Lymphoma Id to a bsAb. The tumor model used was the murine lymphoma A20, which is derived from BALB/c mice and expresses IgG2a. A20 is a suitable model, because it reflects several biological and immunological criteria of clinically observed lymphomas (21). As a target antigen on APCs, we chose the Fcγ receptor II. Murine and human FcγRI and FcγRII were shown to efficiently trigger endocytosis and T-cell activation *in vitro* (22–26) and *in vivo* (27) when targeted with Ig- or bsAb-redirected antigens without the need for adjuvants. In contrast to the high-affinity FcγRI, bsAb binding to FcγRII should not be competed for by endogenous mouse Ig. B cells and neutrophils also bear FcγRII, but they are underrepresented in the peritoneal cavity, which was used as the immunization site.

A prerequisite for using the bsAb-producing hybridoma/tumor hybrids ("triomas") as a cell-based vaccine is the preferential assembly between heavy and light chains of corresponding specificities that is found when the parental mAbs have different species origin (18). This phenomenon also obviates the need for cumbersome *ex vivo* production and purification of the bsAb protein. A20 cells were fused to the rat hybridoma 2.4G2 secreting an antimouse FcγRII IgG2b mAb. The resulting mouse/rat hybrid cell line BiV produces the correctly paired bispecific construct at high yield, as was predicted on the basis of the species origin of the Igs involved and verified by FACS analysis (Fig. 1). FPLC analysis showed that the correctly paired bsAb constitutes 30–40% of the total secreted Ig. bsAb is also exposed on the surface of the trioma cells. BiV cells as well as the parent tumor expressed MHC classes I and II but no detectable B7–1 and low levels of B7–2 molecules on the surface, as was shown by FACS.

Immunization with Purified bsAb *versus* bsAb-producing Cells. In a first step, we tested the immunizing effect of soluble BiV bsAb purified from culture supernatants. BALB/c mice were injected i.p. with BiV bsAb and challenged with A20 cells. However, only a modest survival benefit was observed after preimmunization (Fig. 2*A*). Therefore, other groups of mice were treated i.p. with live BiV cells. Whereas 100% of the animals succumb to 10^5 A20 cells by day 45, the same dose of BiV is not tumorigenic (not shown). Obviously, the trioma cells are eliminated rapidly by virtue of their xenogeneic moiety. A challenge with wild-type tumor after two immunizations with 10^5 BiV cells showed that the mice had developed a systemic immunity with 100% longtime survivors (Fig. 2*B*). Also, an A20 rechallenge given after an interval of 100 days was rejected successfully in all but one mouse. The tumor protection was dependent on the dose of vaccinating BiV cells and could not be achieved by vaccination using irradiated A20 cells (Fig. 2*B*). Also, BiV cells rendered replication incompetent by irradiation could induce tumor immunity (not shown). Furthermore, the antitumor effect was specific, because the outgrowth of another syngeneic B-cell lymphoma (MPC11) could not be delayed by BiV treatment (not shown).

To rule out the possibility that the protection effect of BiV is due solely to its xenogeneic nature, the experiments were repeated using as a vaccine the BiVneg variant. These cells originated from the same fusion as BiV, but they secrete a bsAb, the APC binding arm of which is defective (Fig. 1*C*). Vaccination with these cells resulted in no complete tumor protection (Fig. 2*C*). This indicates that xenogenization alone is not sufficient to induce tumor immunity in 100% of the mice, and that there must be a specific effect mediated by targeting the tumor Id toward APCs. The much higher efficiency of BiV cells in comparison to soluble BiV bsAb might be explained by immunization against other TAAs that are released from the hybrid tumor cells upon their lysis. Thus, synergistic effects may arise provided the immunizing bsAb be delivered in the form of bsAb-producing cells.

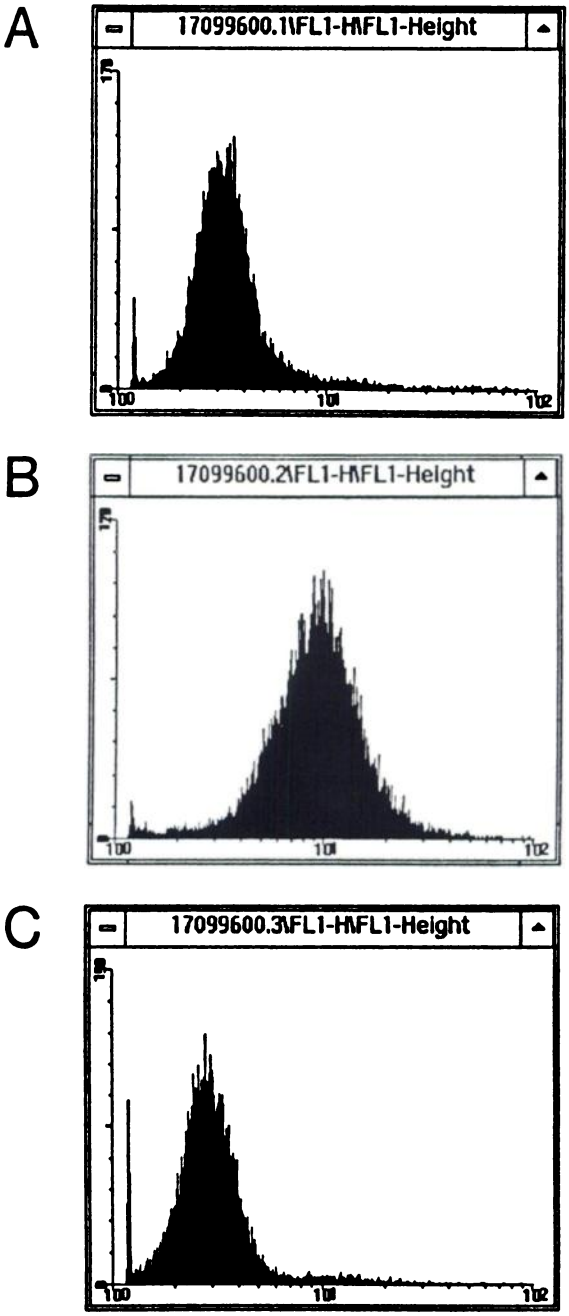

Fig. 1. Characterization of the BiV bsAb. Fcγ receptor-bearing S49 (Thy-1$^-$) cells were incubated with an irrelevant supernatant (*A*), BiV supernatant (*B*), or BiVneg supernatant (*C*). Binding of the bsAb via its rat (2.4G2) arm and the presence of the murine (A20) moiety were verified by FITC-labeled antimouse IgH chain (*B*). Detection by antimouse Igκ chain yields similar results (not shown).

Protection from Simultaneously Injected Tumor Cells. We then asked the question whether BiV treatment is also able to eradicate simultaneously injected wild-type lymphoma cells. BALB/c mice were injected with a mixture of 10^7 BiV cells and a lethal dose of A20 cells. Also in this setting, a significant survival benefit was obtained

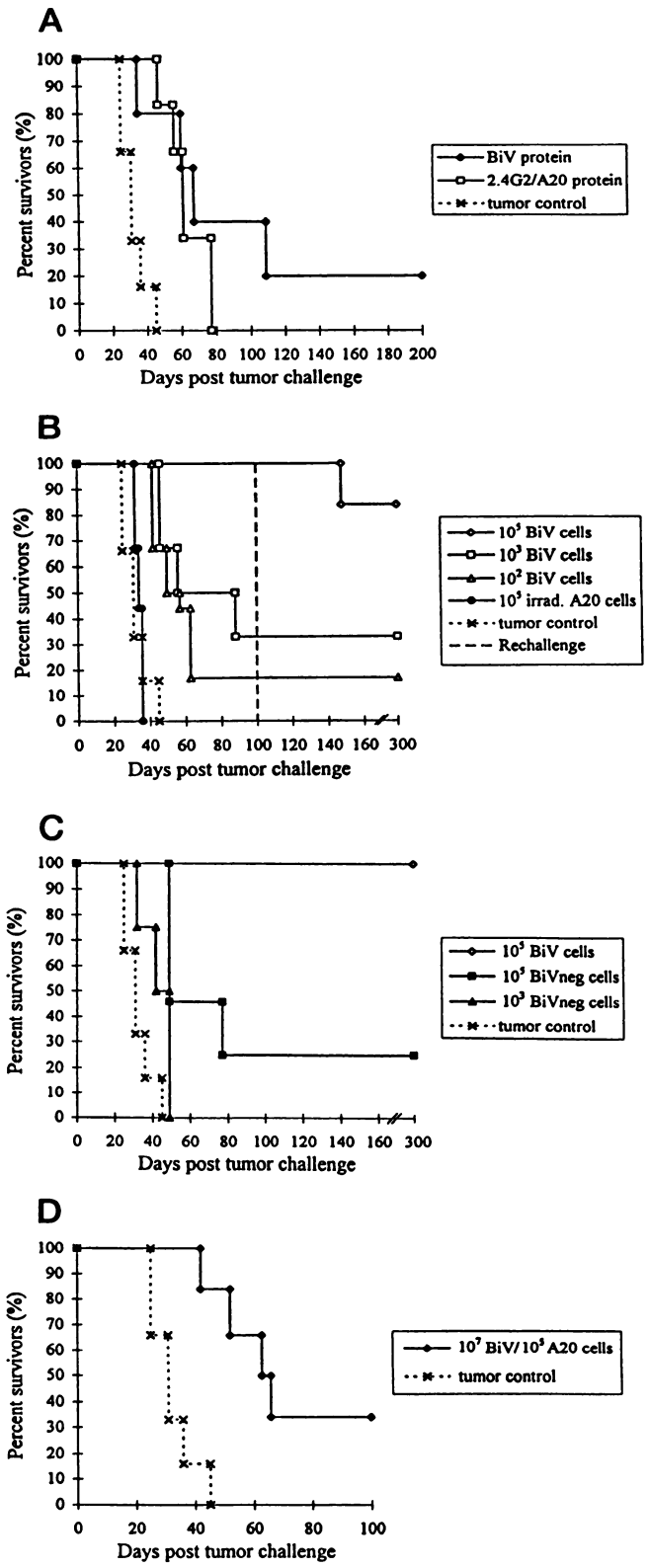

Fig. 2. Protection from A20 lymphoma cells in mice vaccinated with BiV bsAb or BiV cells and challenged with 10^5 A20 cells. In each panel, a control group without immunization is included. Representative results from at least two independent experiments are shown. Details of the immunization procedure are given in "Materials and Methods." *A*, immunization with soluble bsAb. Mice received per injection 50 μg of the purified BiV bsAb or a mixture of 25 μg each of the parental 2.4G2 mAb and the purified A20 IgG2a. The difference between the group receiving the Ab mixture and the tumor control group is not significant ($P = 0.064$). *B*, immunization with viable BiV cells at various doses or 10^5 irradiated A20 cells. Surviving animals received an A20 rechallenge on day 100 (- - - - -). *C*, immunization with BiVneg cells. For comparison, the survival of BiV cell-treated animals is also shown. *D*, survival of animals simultaneously injected with 10^7 BiV cells and 10^5 A20 cells without preimmunization.

($P = 0.022$) as compared to the unimmunized tumor control group, with 33.3% of the animals remaining tumor free for more than 100 days (Fig. 2*D*).

Mechanisms of Tumor Protection. To test for the presence of a humoral anti-Id response, mice vaccinated with BiV cells were bled on the day of A20 inoculation. No or only very low Ab titers could be detected in the immune sera using an anti-idiotypic ELISA (Fig. 3). Similar results were obtained when the unspecific response against A20 cells was determined. The mechanisms of Id-related lymphoma rejection have been controversially discussed. Some reports have demonstrated the capability of immune sera to impart systemic tumor immunity in passive transfer assays (12, 16, 28), but also, lymphoma-specific T-cell responses have been found in protected mice (14, 12, 21, 28). A role of cytotoxic antibodies for mediating the antitumor effect in our model is unlikely because of the absence of reproducible and significant Ab titers but cannot be excluded. If cytotoxic antibodies are involved in tumor rejection, low Ab concentrations seem to be sufficient.

To shed additional light on the protection mechanisms and to dissect the cellular subsets involved in tumor rejection, mice were depleted of $CD4^+$ or $CD8^+$ T cells prior to or following immunization with BiV cells and challenged with A20 cells. The antitumor effect of BiV cells was abrogated in mice that had been depleted of $CD4^+$ or $CD8^+$ cells before immunization (Fig. 4*A*). These data indicate an absolute requirement of both $CD4^+$ and $CD8^+$ cells for the induction of immunity. When the animals were depleted after immunization (Fig. 4*B*), the protective effect was also impaired, but about 50% of the animals survived for more than 80 days. We assume that T cells are instrumental also during the effector phase, but that those T cells that are activated after BiV treatment can escape the mAb-mediated depletion, as was shown previously for $CD4^+$ T cells (29).

Conclusions. The described strategy is a simple method of anti-Id vaccination in that it circumvents the isolation of the Id from the lymphoma cells at the genetic or protein level, which is required in a variety of active Id vaccination protocols described thus far (see, *e.g.*, Refs. 12–17). Our approach takes advantage of the lymphoma Id being a molecularly defined and tumor-specific antigen and obviates the need of gene transfer. The strategy might be applicable to human low-grade lymphomas and plasmacytomas in a minimal residual disease situation. Preferential heavy/light chain pairing, which may be crucial for our cell-based vaccine approach, is also conceivable with Ig from human lymphomas. In general, such tumors are difficult to

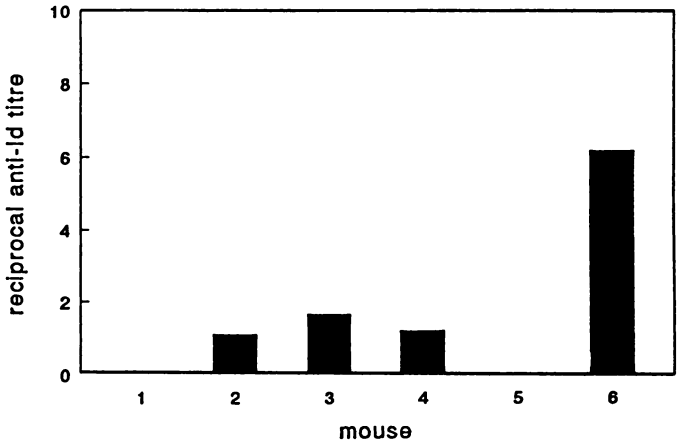

Fig. 3. Anti-idiotypic humoral response in mice protected from the A20 tumor. The results are expressed as those reciprocal Ab titers that yield 2-fold extinction above background.

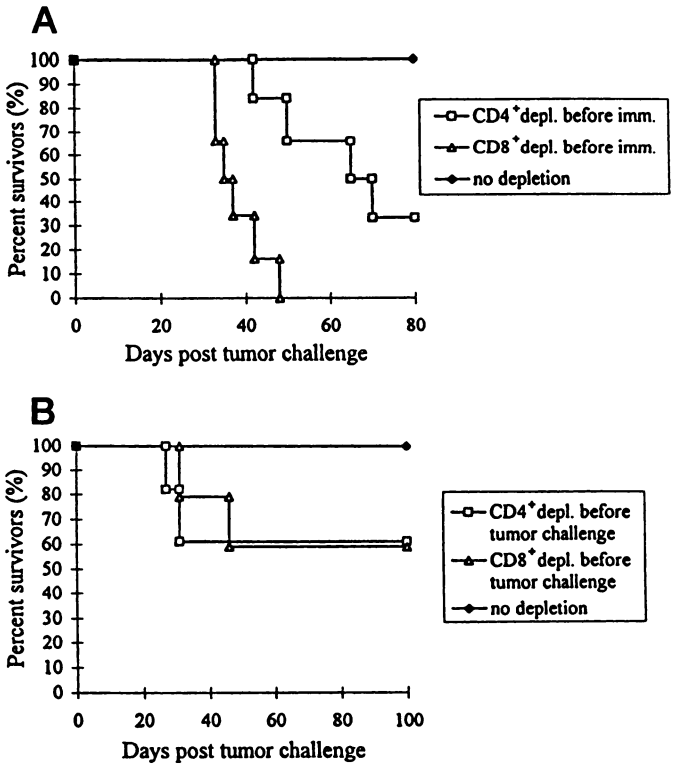

Fig. 4. Protection from the A20 lymphoma in mice vaccinated with BiV cells and depleted of T-cell subsets before BiV immunization (*A*) or before A20 inoculation (*B*). A control group that was immunized with BiV cells and challenged with tumor cells, but which was not depleted of T cells, is also included.

adapt to cell culture. In contrast to genetic modification, however, the fusion of malignant cells isolated from the patient does not require cycling cells. Lymphoma cells fused to the anti-APC hybridoma can be reinjected into the patient without the need of purification of the bsAb. For ethical reasons, the hybrid cells can be irradiated before injection, but this is not necessary, as we have shown.

We have targeted Fc receptors as a model antigen on APCs. In a clinical setting, it might be advantageous to use other APC surface antigens to avoid targeting of B cells. A suitable antigen triggering endocytosis might be the mannose receptor. In summary, the trioma-based vaccination is a promising new approach for the immunotherapy of B-cell malignancies, which might be applied in an adjuvant setting, *i.e.*, after reducing the tumor burden by conventional means.

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