Bispecific Antibodies Increase T-Cell Stimulatory Capacity in Vitro of Human Autologous Virus-modified Tumor Vaccine

Claudia Haas, Gudrun Strauß, Gerd Moldenhauer, Ronald M. Iorio, and Volker Schirrmacher

Divisions of Cellular Immunology [C. H., V. S.] and Molecular Immunology [G. S., G. M.], German Cancer Research Center, 69120 Heidelberg, Germany, and Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01605 [R. M. I.]

ABSTRACT

The production and functional testing of two new bispecific (bs) hybrid antibodies [Abs; bs Ab hemagglutinin-neuraminidase (HN) × CD3 and bs Ab HN × CD28] designed for cancer vaccine modification are described. They allow distinct modifications of the human tumor cell vaccine ATV-NDV, an autologous tumor cell vaccine already modified by infection with Newcastle disease virus. The bs Abs use the viral HN molecule as a common foreign anchoring molecule for attachment to the tumor cells and allow the introduction of anti-CD3 or anti-CD28 T-cell-stimulatory molecules. The bs Abs attached to tumor target cells were able to cross-link CTL effector cells and up-regulate T-cell activation markers on autologous cancer patient-derived CD4 and CD8 T lymphocytes. This strategy of combining a cellular vaccine with a bs Ab is highly specific, quick, and economical and has broad-range applications. Five ng or less of target cell-bound bs Ab HN × CD28 were effective at augmenting T-cell-mediated antitumor cytotoxicity.

INTRODUCTION

The avian paramyxovirus NDV is being used as an antineoplastic agent because of its many antineoplastic (1) and immunopotentiating (2) properties. A lytic substrain was used to generate effective melanoma oncolysates (3). The same strain was shown to be able to induce complete regression of various human tumor types xenotransplanted into nude mice (4), whereas in Hungary, another substrain is being applied to advanced cancer patients to cause nonspecific immunostimulation (2). In the past, we have used the nonlytic, avirulent strain Ulster to produce a virus-modified live tumor cell vaccine (5). In various animal tumor models, such NDV-modified ATV (ATV-NDV) was applied in tumor-bearing animals for ASI and was found to be effective in protecting against micrometastatic disease (6–8) and to establish systemic-specific antitumor immunity (9). An analysis of the mechanism of function revealed at least two important components: (a) the introduction of the viral HN molecule into the cell surface as a new adhesion molecule facilitating lymphocyte-tumor cell interactions (10, 11); and (b) the local induction by NDV of type I IFNs, which are important cytokines for the generation of tumor-specific CTLs (12). Remarkably, the virus modification required only cell surface modification, but not infection (13). It did not change the specificity of the antitumor response and selectively increased the frequency of tumor-specific cytolytic T cell precursors (14).

In this study, we succeeded in generating two bs Abs that bind with one arm to HN of ATV-NDV and with the other arm to defined cell surface molecules of T lymphocytes to cause their activation. These two reagents have a broad range of applications, because they can attach to any NDV-modified vaccine. Contact of the ATV-NDV-bs Ab vaccine with T lymphocytes can cause their activation via either the T-cell receptor-associated CD3 molecule (signal 1 pathway) or CD28 costimulation (signal 2 pathway). This manuscript describes the generation of bs HN × CD3 and bs HN × CD28, their purification and characterization, and their activity in a bs Ab vaccine.

MATERIALS AND METHODS

Cell Lines and Isolation of Hybrid Hybridoma. The hybridoma 15E8 (CD28/IgG1) was kindly provided by Dr. R. van Lier (Netherlands Center for Blood Transfusion, Amsterdam, the Netherlands). The hybridoma OKT3 (CD3/IgG2a; κ) was obtained from the American Type Culture Collection (Rockville, MD), and the hybridoma HN1,4 c (IgG2a; κ) was obtained from co-author R. M. I. (15). All hybridoma cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Eggenstein, Germany) supplemented with L-glutamine (2 mM), penicillin (50 IU/ml), streptomycin (50 μg/ml), and 10% FCS (referred to as complete medium).

15E8 and OKT3 hybridoma cells were selected for hypoxanthine-guanine phosphoribosyltransferase deficiency by culture in 8-azaguanine (0.13 μM)-containing medium. They were then subcloned several times. The HN1,4 c hybridoma was subcloned three times, until we had established the good Ab-producing subclone HN.B. Before cell fusion, HN.B cells were pretreated with a lethal dose of iodoacetamide (5 mM; 30 min, 4°C). Cell fusion was carried out using a standard protocol (16).
Briefly, $4 \times 10^7$ cells of each parental line were washed twice in RPMI 1640, mixed, and then fused by the addition of pre-warmed (37°C) polyethylene glycol 1500 (Boehringer Mannheim). Hybrid hybridoma cells were grown in complete medium supplemented with selection medium containing HAT. Supernatants were checked for the presence of correct immunoglobulin binding activity using either NDV-infected tumor cells or CD3/CD28-expressing cells as a binding partner and flow cytometry for detection. In the case of the HN×CD28 hybrid hybridoma, individual wells were tested for the presence of both parental isotypes. Multiple subcloning was performed to establish stable Ab-producing lines.

The human colon carcinoma cell line Colo-205 and the Jurkat T-ALL cell line (CD3⁺ CD28⁺) that were used as binding partners were also cultured in complete medium.

**Screening Assay for bs Abs.** Determination of bs anti-HN × anti-CD28 activity was performed by flow cytometry analysis as follows: NDV-modified Colo-205 cells ($1 \times 10^6$) were incubated with hybridoma supernatants, elution fractions, or purified bs Ab diluted with culture medium for 30 min at 4°C. After several washing steps, the reaction was developed using either PE-labeled goat antimouse IgG1 or PE-labeled goat antimouse IgG2a Abs (Biozol, Eching; Germany). Flow cytometric analysis was performed on a FACSscan analyzer (Becton Dickinson, Heidelberg, Germany) with FACSscan software according to the manufacturer’s recommendations.

**Purification of Abs.** Hybrid hybridomas were grown in mini fermenter (miniperm; Heraeus, Osterode, Germany; Ref. 17). The production module contained complete medium, whereas the supply module contained RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin, and 5% FCS. Harvested Abs were first purified over a protein G column (Pharbiotigs, Nurtingen, Germany) and dialyzed against 10 mM morpholino-ethanesulfonic acid (pH 5.6; Serva, Heidelberg, Germany). The final volume was 200 μl. The plates were incubated for 4 h at 37°C in a CO₂ incubator. The specific $^{51}$Cr release was calculated according to the following formula:

\[
\text{Percentage of specific release} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100
\]

**RESULTS**

**Production, Purification and Characterization of the New bs Abs HN×CD28 and HN×CD3.** The bs Abs were produced by the hybridoma technology in HAT selection medium. The fusion partners anti-CD28 (IgG1; κ) and anti-CD3 (IgG2a; κ) were first grown in medium containing 8-azaguanine (0.13 μm) to generate resistant variants that cannot grow in the HAT selection medium. The HAT-resistant HN.B hybridoma (IgG2a; κ) was treated with a lethal dose of iodoacetamide just before fusion, so that no viable nonfused cells could survive. After polyethylene glycol-mediated cell fusion, quadroma were selected in HAT medium, and supernatants were tested for the presence of correct immunoglobulin binding activity using either NDV-infected tumor cells or CD3/CD28-expressing cells as the binding partner and FACS flow cytometry for detection. For both quadromas, about 10 of 600 wells tested were reactive with both respective target cells. Multiple subcloning was performed to establish stable Ab-producing lines.

For purification, concentrated supernatant collected from the production module of a mini fermenter was first purified over a protein G column and then given over an HPLC ion exchange column. Fig. 1 shows the HPLC elution profiles for bs Ab HN×CD28 (Fig. 1A) with three peaks and for bs Ab HN×CD3 (Fig. 1B) with five peaks. Fractions collected at 30-s intervals were tested for bispecificity by FACS analysis (see below; Fig. 2), and the peaks containing the highest reactivity (shaded area in Fig. 1, A and B) were pooled. Fig. 1C shows the results from SDS-PAGE analysis of parental mAbs and hybrid Abs under reducing conditions from a 10% gel. Lane 3 shows the bs Ab HN×CD28 with two different L and H chains that differ markedly from each other. The H chain with the higher molecular weight originated from HN.B, and the other one originated from...
CD28. With regard to bs Ab HN×CD3, no differences could be seen between H or L chains.

The bispecificity of the purified quadroma batches was tested by FACS analysis using as target cells the FC-γ receptor-negative human colon carcinoma line Colo-205 with or without NDV infection and the Jurkat T-ALL-cell line selected for high expression of CD3 and CD28. Results are shown in Fig. 2. mAb HN×CD28 bound to NDV-infected Colo-205 tumor cells (Fig. 2A) but not to noninfected Colo-205 tumor cells and also bound to CD28-positive Jurkat cells. Because this bs Ab contains two different isotypes, binding could be demonstrated by PE-labeled goat antimouse IgG1-specific Abs (dotted lines) as well as by PE-labeled goat antimouse IgG2a-specific Abs (continuous lines; differences in staining intensities could be due to differences in the strength of the second Ab or to the presence of additional IgG1). The results demonstrate the bispecificity of bs Ab HN×CD28. When NDV-infected Colo-205 cells (Fig. 2D) or CD3⁺ Jurkat cells (Fig. 2F) were incubated with bs Ab HN×CD3, they could be stained with PE-labeled goat antimouse IgG2a, whereas noninfected Colo-205 cells (Fig. 2E) did not bind the quadroma and therefore remained unstained.

The purified bs Abs were further characterized in functional tests before they were used to modify tumor vaccines. Induction of proliferation by PBMCs was tested in a conventional [³H]thymidine uptake assay, using PHA as positive control and medium alone as negative control (Fig. 3). At different times after stimulation with 1 μg/ml reagent, the proliferative capacity was tested. bs Ab HN×CD3 had a stimulatory capacity over time similar to that of PHA. Because it is known that such activity requires cross-linking, it is likely that the Fe portion of the Ab bound to Fe receptors on monocytes. The same could be true for the results obtained with bs Ab HN×CD28, which was slightly less effective. A combination of both bs Ab reagents led to the strongest stimulation of proliferation, with a maximum at day 4. These results have thus demonstrated agonistic and synergistic functional activity of the two bs Abs, a prerequisite for the experiments to follow in which the reagents are used for cell surface modification of tumor vaccines.

bs Abs Attached to Tumor Cells Cross-Link CTL Effector Cells: Specificity, Dose Dependency, and Kinetics. An important proof for bispecificity of the isolated Ab is a functional assay in which it can be shown that cells expressing the two different target molecules can be cross-linked. The two bs Ab reagents were therefore tested for their ability to cross-link preactivated T cells that express CD3 and CD28 with NDV-modified tumor cells. Such cross-linking should lead to increased tumor cell lysis. Effector T cells from healthy donors were generated by in vitro stimulation of PBLs with IL-2. As shown by the results in Fig. 4A, these effector cells could lyse...
NDV-infected Colo-205 tumor cells about two times better when these were precoated with bs Ab HN×CD3 or HN×CD28 in comparison to tumor cell lysis without bs Abs. A specificity control consisting of noninfected tumor cells that could not bind bs Abs showed no augmented lysis (Fig. 4B). Fig. 4C shows the results obtained when bs Abs, attached to NDV-infected Colo-205 cells, were used not only as targets, but also as stimulator cells in a MLTC. In the five experimental groups, T lymphocytes were stimulated either with irradiated NDV-infected Colo-205 (TuN; I) or with TuN to which HN×CD3 (II) or HN×CD28 (III) or both (V) had been attached. IV represents a control group in which the TuN stimulator cells had been preincubated with the three parental mAbs and then washed. The black columns show the cytotoxic activity of the generated effector cells as tested in a subsequent 4-h CTL 

51Cr release test with NDV-infected Colo-205 target cells. The gray columns show the respective target cell lysis of NDV-infected Colo-205 cells that had been additionally coated with bs Ab HN×CD3 and/or HN×CD28. The increased lysis represented by the gray in comparison to the black columns shows the additional effect of cross-linking of effector cells to target cells. In the MLTC cultures, stimulator cell modification by bs Ab showed a strong augmenting effect only when both bs Abs were combined (V). When the stimulator cells were modified by only one bs Ab (II and III) or with the three parental mAbs (IV), such washed stimulator cells were not able to augment CTL activity. Similarly, when Colo-205 cells were used that were not preinfected with NDV, the modification with bs Ab also had no effect in the

Fig. 2. FACS cytometric analysis of dual binding specificity of bs Ab. A–C, bs Ab HN×CD28 is shown to bind to two different targets: NDV-infected (A) but not uninfected (B) Colo-205 tumor cells and CD28⁺ Jurkat T-ALL cells (C). After washing, bound Abs were revealed by a second incubation with phyceroerythrin-conjugated goat antimouse IgG1-specific Abs (dotted line) or with goat antimouse IgG2a-specific Abs (solid line). D–F, bs Ab HN×CD3 is shown to bind to two different targets: NDV-infected (D) but not uninfected (E) Colo-205 tumor cells and CD3⁺ Jurkat T-ALL cells (F). After washing, bound Abs were revealed by a second incubation with phyceroerythrin-conjugated goat antimouse IgG2a-specific Abs.
MLTC-CTL system (data not shown). Thus, these results show that stimulator cell modification with bs Ab requires specific Ab binding via HN, and also that anti-CD3 and anti-CD28 signals are required to induce CTL effector cells in MLTC.

To define the minimal effective dose of bs Ab, a titration was performed in an assay analogous to Fig. 4A, in which NDV-infected Colo-205 target cells were precoated with decreasing amounts of bs Ab, washed, and then added to IL-2-activated PBLs as effector cells. As shown by the results in Table 1, bs Ab HN×CD28 reached its maximal effect at a protein concentration of 0.1 μg/ml, whereas bs Ab HN×CD3 had its maximum effect at 10 μg/ml. At 0.01 μg/ml, the functional activity started to decrease with both bs Abs. In additional assays, a standard concentration of 1 μg/ml was used as a minimal saturating dose. This corresponds to 50 ng of protein per 10^6 target cells.

To test how fast bs Ab-mediated lysis occurs, bs Ab-coated 51Cr-labeled NDV-infected tumor cells were added to preactivated effector cells (Table 2). Whereas after 1 h, there was only a slight increase of cytotoxicity, after 2 h, it was 3-fold, after 3 h, it was 5-fold, and after 4 h, it was 7-fold.

bs Ab Attached to the Cell Surface of ATV-NDV Augments Its T-Cell Stimulatory Capacity. Another functional test for the bs Abs consisted of testing activation of resting T cells via up-regulation of the T-cell activation markers CD69 and CD25 (IL-2 receptor α chain). In the experiment shown in
Fig. 5. PBLs from a normal healthy donor were stimulated in allogeneic MLTC with HT 29 colon carcinoma cells that were first infected with NDV and then coated with bs HN×CD3 or bs HN×CD28 at the concentrations indicated. The stimulator cells were mixed 1:1 to provide both anti-CD3 and anti-CD28 stimuli and to achieve synergistic and optimal stimulation, as in the CTL induction experiment (Fig. 4). After stimulation for 1, 3, or 5 days, the CD3 T cells were double-stained for expression of CD69 (Fig. 5A) or CD25 (Fig. 5B). Maximal up-regulation of CD69 was observed 1 day after stimulation, when 65–85% of the CD3 T cells were positive. When using 1 ng/ml bs Ab for modification, this peak activation was followed by a decrease to 50%, whereas with higher Ab concentrations, CD69 up-regulation remained at a plateau level. The kinetics of CD25 up-regulation were similar when using the higher bs Ab concentrations. When using 1 ng/ml, however, the kinetics were different, reaching peak activity only at day 3.

The most critical and essential in vitro test for effectiveness of the bs Ab modified tumor vaccine was an assay in which cancer patient-derived lymphocytes were coincubated with vaccines derived from their own operated tumor. Fig. 6 illustrates the results of such ATV-NDV-bs Ab stimulation experiments with PBLs from two cancer patients. At the top of Fig. 6, T-cell activation is shown for a patient with colorectal carcinoma at Duke’s D stage with an operated liver metastasis. The tumor cells used in this experiment were in their fourth passage from a primary culture of the liver metastases. They had a very undifferentiated morphology. These DAT 45 cells were used as stimulator cells after NDV infection (TuN) or after modification with bs HN×CD28, or the cultures were stimulated with cells modified by each of the two bs Abs and then mixed 1:1. Whereas before stimulation (day 0), no double-positive T cells were observed, the stimulation with TuN alone caused up-regulation of CD69 on a small fraction of CD8 and CD4 T lymphocytes at day 1 but not at day 3. A much stronger and longer-lasting up-regulation of CD69 was observed with TuN-HN×CD28, whereas the stimulation with both bs Ab modified vaccines caused a maximal effect, with about two-thirds of the cells stimulated over 3 days. Also, up-regulation of CD25 was observed, but the kinetics were delayed, and the effects seen with TuN-HN×CD28 were only half-maximal. When using the parental anti-HN mAb alone or unmodified autologous tumor cells for stimulation, no up-regulation of T-cell activation markers was seen in these autologous MLTCs. The second patient tested had glioblastoma grade IV, a malignant tumor with a high degree of anaplasia. Standard therapy consists of surgery combined with chemotherapy and radiation therapy. Nevertheless, postoperative survival, on the average, is not more than 6 months. Such tumors often produce immunosuppressive factors. This could account for the fact that CD8 T cells were only 9% as compared to 40–50% in normal PBLs. The percentage of CD4 T cells was in the normal range. In spite of this, principally similar results of T-cell activation were observed after the various tumor cell modification procedures. The NCH 92 tumor cells from primary cultures were γ-irradiated, NDV-infected, coated with the indicated Abs, washed, and added at a ratio of 1:10 (tumor cells:PBLs) to the autologous PBLs. As can be seen from the bottom of Fig. 6, NDV infection alone led to up-regulation of CD25 on CD4 T cells at days 1 and 3 and to CD69 up-regulation on a small proportion of CD8 and CD4 T cells. The bs Ab-coated autologous vaccine ATV-NDV caused up-regulation within 24 h of all activation markers on all investigated subpopulations of T cells. Interestingly, the vaccine TuN-HN×CD28 alone was as active as the vaccine containing both bs Abs, except for the CD8+/CD45− subpopulation. When unmodified NCH 92 tumor cells or NDV-infected cells coated with the parental mAb HN.B were used, no T-cell stimulation could be observed with patient-derived lymphocytes (data not shown).

**DISCUSSION**

In recent years, the concept of costimulation for generating effective T-cell-mediated immunity has become of major importance (19–21). The effects that costimulatory molecules [e.g., CD80/CD86 (B7.1/B7.2)] and their receptors (e.g., CD28/CTLA-4) can have on immune responses in vivo were demonstrated in various model systems (for review, see Refs. 22 and 23). The presentation of anti-CD3 and anti-CD28 mAbs to T lymphocytes by melanoma tumor cells via bs and bivalent antimalanoma heteroconjugates caused T-cell proliferation and potent tumor cell lysis (24). Whereas these early experiments

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**Table 1** Efficacy of bs Abs to cross-link target cells to effector cells and to mediate cytolysis

<table>
<thead>
<tr>
<th>Concentration of bs Ab</th>
<th>% specific lysisa in the presence of</th>
<th>bs HN×CD3</th>
<th>bs HN×CD28</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µg/ml</td>
<td></td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>1 µg/ml</td>
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<td>95</td>
<td>75</td>
</tr>
<tr>
<td>0.1 µg/ml</td>
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<td>86</td>
<td>83</td>
</tr>
<tr>
<td>0.01 µg/ml</td>
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<td>86</td>
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* Time of 51Cr release assay after coincubation of target cells with IL-2-preactivated human PBLs as effector cells.

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**Table 2** Kinetics of TuN-bs Ab (Colo-205-NDV-HN×CD3) target cell lysis

<table>
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<tr>
<th>Time (h)</th>
<th>50:1</th>
<th>25:1</th>
<th>12:1</th>
<th>6:1</th>
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<tr>
<td>4</td>
<td>69</td>
<td>60</td>
<td>49</td>
<td>29</td>
</tr>
</tbody>
</table>

* Time of 51Cr release assay after coincubation of target cells with IL-2-preactivated human PBLs as effector cells.
used chemically cross-linked mAbs, Möller and Reisfeld (25) generated bs Ab by the fusion of two respective hybridomas. The hybrid hybridoma technology of generating quadromas, which was also used in this study, offers some advantages over chemical cross-linking. Once a hybrid hybridoma is established, it is a source of continuous production of the bs Ab. bs Abs produced via the hybridoma technology have been reported to have a longer half-life in the circulation and a better ability to penetrate to extravascular sites, and they lack heteroaggregates that are often found in chemically cross-linked constructs that can damage vital organs such as the kidney (26).

To date, there are already a number of publications about bs Abs: many from in vitro studies (for review, see Ref. 26); some from preclinical tumor model systems (27-29); and a few from clinical studies (30-33). Most therapeutic strategies against tumor cells using bs Abs have designed and used bs reagents that recognize the CD3 molecule on T cells (34-36), Fc receptors on macrophages (37, 38), or natural killer cells (39) with one arm and TAAs with the other arm. Thus, for each tumor type, distinct reagents have to be generated. It is apparent from clinical data that antitumor Abs that cross-react with normal tissue will generate unwanted side effects (40). Another problem with the clinical application of bs Abs consists in avoiding unspecific activation and depletion of T cells, especially when the bs constructs such as CD3×TAA or CD28×TAA are given systemically. For this reason, in many in vivo applications (animal models or clinical trials), the lymphocytes are first activated ex vivo with IL-2 and then loaded with bs Ab and reinfused. Renner et al. (41), however, reported therapy effects after i.v. injection of bs Ab without preloading of either tumor cells or lymphocytes in a SCID mouse model.

We present here an entirely new concept that may circumvent some of the above-mentioned problems. Our strategy combines bs Abs with a tumor vaccine to improve the vaccine immunogenicity for active specific immunization. In animal models (6-10) as well as in previous Phase I and II clinical studies (5, 42-44), we established a new type of ATV modified by infection with NDV (ATV-NDV). For its preparation, a standard protocol was worked out that involves isolation of autologous tumor cells from freshly removed tumor tissue by dissociation, purification, and cryopreservation, followed by γ-irradiation and infection by NDV (43). Such ATV-NDV vaccines were applied intradermally, and the effects of such ASI were evaluated. Promising results from clinical Phase II studies applying an ATV-NDV vaccine have already been reported for renal carcinoma (44), colorectal carcinoma (42, 43), breast carcinoma (45), and ovarian carcinoma (45).

Here, we report on our success in producing and purifying two new functionally active bs Abs that were specially designed for ATV-NDV vaccine modification. bs HN×CD28 and bs HN×CD3 were shown to attach to NDV-modified tumor cells but not to nonmodified tumor cells, thus demonstrating their expected specificity. In the functional assays, each bs Ab was administered as it would be in a tumor vaccine; this means that the tumor cells were infected by NDV according to the established clinical standard protocol and then incubated for 20 min at 4°C with bs Ab, followed by washing to remove unbound material. The vaccine was shown to cross-link human (preactivated) effector T cells with human tumor cells and to mediate augmented tumor lysis. As little as 50 ng of protein represented a minimal saturating dose for 10⁶ tumor target cells. For induction of CTL activity, synergistic stimulatory effects were described in vitro when using a 1:1 combination of TuN-bs HN×CD3 and TuN-bs HN×CD28 vaccine. Within 1 day of stimulation of resting human T lymphocytes by colon carcinoma cells thus modified, 65-85% of CD3 T cells expressed CD69 activation markers. This would mean that 50 ng of bs Ab protein bound to tumor cells and thus focused within a vaccine could possibly activate a major fraction of contacting T lymphocytes within 24-48 h. Usually, for clinical application, much higher Ab concentrations are required.

The ATV-NDV vaccine modified by bs Ab HN×CD28 enables tumor antigen and costimulator to be expressed on the same cell surface, a prerequisite for an optimal T-cell response.
728 T-Cell Stimulation via Vaccine-coupled bs Abs

**A**

<table>
<thead>
<tr>
<th>% double positive cells</th>
<th>days after stimulation</th>
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<tr>
<td>CD8/CD25</td>
<td>0</td>
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<tr>
<td>CD4/CD25</td>
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**B**

<table>
<thead>
<tr>
<th>% double positive cells</th>
<th>days after stimulation</th>
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<tr>
<td>CD8/CD69</td>
<td>0</td>
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<tr>
<td>CD4/CD69</td>
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**Fig. 6** Activation of cancer patient-derived T lymphocytes with autologous bs Ab modified tumor vaccines in MLTCs in vitro. A. PBLs from a patient with metastasized colon carcinoma (stage Duke’s D), operated on with curative intent from the primary tumor and a solitary liver metastasis. The percentage of double-fluorescence positive CD4 and CD8 T cells before (day 0) and after (days 1 and 3) stimulation was determined by FACS analysis. TuN bs Ab: □. TuN (DaT45-NDV); □. TuN-HN×CD28; □. TuN-HN×CD28 + TuN-HN×CD3. B. PBLs from a patient with glioblastoma (grade IV) were stimulated with modified autologous NCH 92 tumor cells, HN XCD28: □. TuN-HN×CD28 + TuN-HN×CD3.

Such a vaccine may be comparable in immunogenic strength to tumor cells double-transfected with a cytokine gene and CD80, which were reported to be strong vaccines and to induce CD25 in tumor-infiltrating lymphocytes (47). Agonistic anti-CD28 Abs may be better suited for costimulation than CD80, because unlike the latter, they do not interact with CTLA-4, which mediates a negative signal (48).

Although several studies demonstrated indirect priming of T cells by tumor cells via host-derived antigen-presenting cells (49), direct costimulation of T cells by B7-transfected tumor cells was also shown (50, 51). Furthermore, we reported that tumor cell membrane integrity and viability were important parameters for CTL activation in vitro and in vivo (52), and from recent clinical studies, we reported that tumor cell viability behaved as an independent factor for vaccine quality and effectiveness (45). These findings thus support the concept of costimulation of T cells by tumor cells as a viable strategy for the generation of potent cancer vaccines.

The new reagents described in this study represent an important new step for further improvement of ATV-NDV vaccines. Although the binding of bs Ab to ATV-NDV blocks the attachment site of HN for augmented cell-cell interactions and costimulation (10, 11), this is more than compensated for by the stronger costimulatory effects provided by the agonistic anti-CD28 Ab. The advantages of this strategy of modification of cellular vaccines can be summarized as follows:

1. **Specificity.** With the viral HN molecule, the procedure uses a common foreign anchoring molecule for attachment of bs Ab to the tumor cells and thus does not interfere with TAA presentation, and it allows the introduction of defined T-cell-stimulatory molecules.

2. **Economy.** The modification procedure is quick and economical, and the whole tumor cell modification procedure of virus infection and loading with bs Ab can be performed within 1 h. The amounts of reagents used are in the range of 1 ng of virus protein and 1 μg of bs Ab protein per vaccine.

3. **Safety.** The nonvirulent NDV strain Ulster that was used cannot replicate in cells other than the in vitro infected tumor cells. After infection, it goes through a monocyclic abortive replication cycle and produces noninfectious virus particles. Therefore, on in vivo, the virus cannot spread to neighboring cells, because only cell surface binding and not virus replication is required for NDV-mediated tumor cell modification; even UV radiation-inactivated NDV can be used (13).

For clinical ASI studies, ATVs offer several advantages compared to allogeneic vaccines and perhaps even to the rapidly expanding library of tumor-associated peptides. They represent the closest possible match to each individual cancer of a patient and contain as many as possible of the potential TAAs. ATV-NDV is composed of > 1.5 × 10⁶ autologous tumor cells that possibly represent the antigenic heterogeneity and multiplicity that most likely characterize a malignant tumor. A polyvalent vaccine like this may have a smaller risk to select for immune escape variants or to induce apoptosis and deletion in responding T cells than a monovalent vaccine. In this context, an interesting report recently appeared in which vaccination with a...
defined adenovirus-derived peptide caused tumor enhancement, whereas vaccination with the complete virus caused tumor protection (53). Our concept of combining bs Abs with ATV-NDV also avoids the logistic problems of somatic gene therapy approaches when applied to autologous tumor cells.

Several modes of application of the bs Abs can be envisaged: in the case of tumor cells expressing TAA but missing costimulatory molecules, the attachment of bs Ab HN×CD28 may be sufficient to prevent a tolerogenic signal. In the case of tumor cells lacking MHC molecules or TAAs or having defects in antigen presentation, an unspecific activation of T cells by a combination of both bs Abs (bs HN×CD3 and bs HN×CD28) may be envisaged, thus leading to an improvement of the microenvironment for the activation of other immune defense and tumor resistance mechanisms. The vaccine ATV-NDV-bs Ab can also be used for \textit{ex vivo} activation of patient-derived PBL T cells. Such autologous activated T cells could be further expanded and used for adoptive cellular immunotherapy. This idea is corroborated by the findings of Flens \textit{et al.} (54), who were able to show that stimulation of tumor-infiltrating lymphocytes with CD3/CD28 mAbs induced significantly stronger proliferation and yielded higher levels of cell recovery than usage of high-dose recombinant IL-2. They observed that during the initial phase of activation, expansion of CD4$^+$ lymphocytes dominated, but within 4 weeks of culturing, the CD8$^+$ population increased to values over 90\%, indicating a large pool of cytotoxic effector cells. An interesting additional aspect consists of combining the two bs tumor vaccines sequentially. It was reported that stimulation of T cells with anti-CD3 Abs augments their expression of CD28 molecules (55). Therefore, to increase the efficacy of vaccination, it might be possible to treat patients first with ATV-NDV-bs HN×CD3 to up-regulate CD28 expression and thereafter with ATV-NDV-bs HN×CD28.

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