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Target cell-induced T cell activation with bi- and trispecific antibody fragments*

Previously we proposed a concept for tumor immunotherapy in which two different bispecific antibody conjugates, an anti-target × anti-CD3 and an anti-target × anti-CD28 conjugate, induce the activation of resting human T cells upon binding to the respective tumor target cells. After *in vivo* application of these reagents, this model of a "target cell-induced T cell activation" envisages the destruction of target cells by *in situ* activated T cells. Obviously however, for *in vivo* application, the use of Fc-free antibody fragments is mandatory to prevent binding of the conjugates to Fc receptor-positive cells which would lead to Fc-mediated T cell activation. Here we report a simplification of published procedures for the generation of bispecific Fab-hybrid fragments, univalent for each antigen. We demonstrate that an anti-target × anti-CD3/anti-target × anti-CD28 combination of such hybrids, as well as an identical combination of covalently coupled $F(ab')_2$ fragments, mediate "target cell-induced T cell activation" in an *in vitro* test system. Thus, these reagents may be capable of inducing an *in situ* activation of human T cells upon systemic *in vivo* application according to the concept outlined above. A trispecific conjugate with anti-target, anti-CD3- and anti-CD28 specificity appears to be unsuitable for this purpose because it activates resting T cells in soluble form without requiring immobilization through binding via its anti-target portion.

1 Introduction

Several years ago work in different laboratories indicated a new and attractive approach to focusing a potent immune effector mechanism with antibodies directed to cellular target antigens: bispecific antibody conjugates consisting of an anti-target antibody and an antibody to the TcR/CD3 complex on T cells were demonstrated to specifically trigger the lysis of the target cells by receptor positive CTL [1–3].

Since human T cells freshly isolated from peripheral blood, in contrast to cloned cells, have little lytic activity [4], even in the presence of conjugates of the type described above [5], we focused our attention on the generation of lytic activity in resting T cells as part of the T cell activation process [5, 6]. This work and the model of a "target cell-induced T cell activation" subsequently derived from it [7, 8] is based upon, and supports, an increasingly accepted notion recently summarized by Janeway [9]. It claims that under physiological conditions the effective activation of resting T cells requires at least two signals, usually provided by APC; signal one being the appropriate triggering of the TcR/CD3 complex by properly presented antigen and signal two being delivered nonspecifically by the APC via receptor(s) for accessory signals on the T cell. Notably, while two signals are necessary to initiate the activation process (*i.e.* the generation of the lytic state), only signal one is required to trigger the effector function of an already activated cell (*i.e.* the delivery of cytotoxicity).

Accordingly, in our model system we proposed the use of two different bispecific antibody conjugates, an anti-target × anti-CD3 and an anti-target × anti-CD28 conjugate, the latter triggering a well known receptor for accessory signals on T cells [10]. By binding the two conjugates, the target cell becomes an APC in the sense described above and induces effective T cell activation. Since the activating function of the anti-CD3-containing conjugate is dependent on its immobilization at the target cell surface, both conjugates, alone or in combination, are not capable of activating T cells in the absence of the target cells. To this end the activation process is target cell induced. Once activated, cytolytic T cells are triggered to lyse the target cells by the anti-target × anti-CD3 conjugate.

Upon *in vivo* application of certain bispecific antibody conjugates, the described model system envisages the *in situ* activation of human T cells, if binding of the reagents through their variable regions rather than their Fc portions is ensured.

In this report we describe the construction of different Fc-free bi- and trispecific antibody fragments and investigate their capability to mediate "target cell-induced T cell activation".

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Abbreviations: DTNB: 5,5'Dithiobis-2-nitrobenzoic acid **SPDP:** N-succinimidyl 3-(2-pyridyldithio)proprionate **TNB:** 5-Thio-2-nitrobenzoate

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2 Materials and methods

2.1 Cells and antibodies

The M21 melanoma cell line was originally provided by D. L. Morton (University of California, Los Angeles, CA). Human PBMC were isolated by density gradient centrifugation with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). In some experiments T cells were purified from PBMC using a commercially available reagent (T-Kwik, One Lambda, Los Angeles, CA). This reagent contains various cytolytic mAb and complement to lyse contaminating cells [11]. All cells were kept in complete growth medium containing RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine and gentamycin at 50 μg/ml. mAb 9.2.27 is directed to a melanoma-associated proteoglycan complex [12], mAb OKT3 (American Type Culture Collection, Rockville, MD) to the CD3- and mAb 9.3 to the CD28-antigen on human T cells. All antibodies are of the IgG_{2a} subtype and were purified from hybridoma SN by affinity chromatography on Sepharose-coupled protein A (Pharmacia).

2.2 Purification and chemical coupling of $F(ab')_2$ fragments

Antibodies were digested with pepsin (Sigma, St. Louis, MO) at a ratio of 30 μg pepsin/mg antibody in a 0.1 M sodium citrate buffer, pH 4.1, containing 0.1 M sodium chloride. Optimal duration of the digestion process was determined by SDS-PAGE to be 22, 18 and 14 h for the 9.2.27, OKT3 and 9.3 antibody, respectively. $F(ab')_2$ fragments were purified from the digest using gel filtration on a Superdex 200 column (Pharmacia). The 9.2.27 $F(ab')_2$ fragment was covalently conjugated to the OKT3 or 9.3 fragment using the heterobifunctional cross-linking reagent N-succinimidyl 3-(2-pyridyldithio)proprionate (SPDP; Sigma) as described [5].

2.3 Evaluation of target cell-induced T cell activation

PBMC or purified T cells (5×10^5/ml) were plated in 96-well microtiter plates (Falcon, Oxnard, CA) with irradiated M21 target cells (5×10^4/ml, irradiated with 100 Gy), and with a 1 : 1 mixture of 9.2.27 × OKT3 and 9.2.27 × 9.3 reagents at different concentrations. After 4 days [^{3}H]dThd (Amersham Int., Amersham, GB) was added to 4 μCi/ml (= 148 kBq) and cells were harvested after 4 h using an automatic cell harvester (Cambridge Technology Inc., Cambridge, MA). Usually in these experiments the culture medium was supplemented with 20% autologous human serum instead of FCS. Two control settings were used: (a) the 9.2.27 × OKT3 reagent alone together with the target cells which serve to immobilize the bispecific antibodies and (b) a 1 : 1 mixture of the 9.2.27 × OKT3 and the 9.2.27 × 9.3 reagent without the target cells.

In some experiments mixed cultures of PBMC and M21 target cells were set up as described above without prior irradiation of the tumor target cells. Viable M21 cells adhere to the culture flask and are distinguishable from lymphocytes due to their large size. Thus, it was possible to assess target cell death easily by light microscopy and trypan blue uptake.

3 Results

3.1 Bispecific conjugates of different $F(ab')_2$ fragments

$F(ab')_2$ fragments were prepared and conjugated as described in Sect. 2.2. Two conjugates were constructed with the specificities 9.2.27 × OKT3 and 9.2.27 × 9.3, respectively (Fig. 1A).

As shown in Fig. 2A a mixture of the two conjugates effectively activated human PBMC in the presence of melanoma target cells, which bind and immobilize the conjugates. No activation was observed if either the

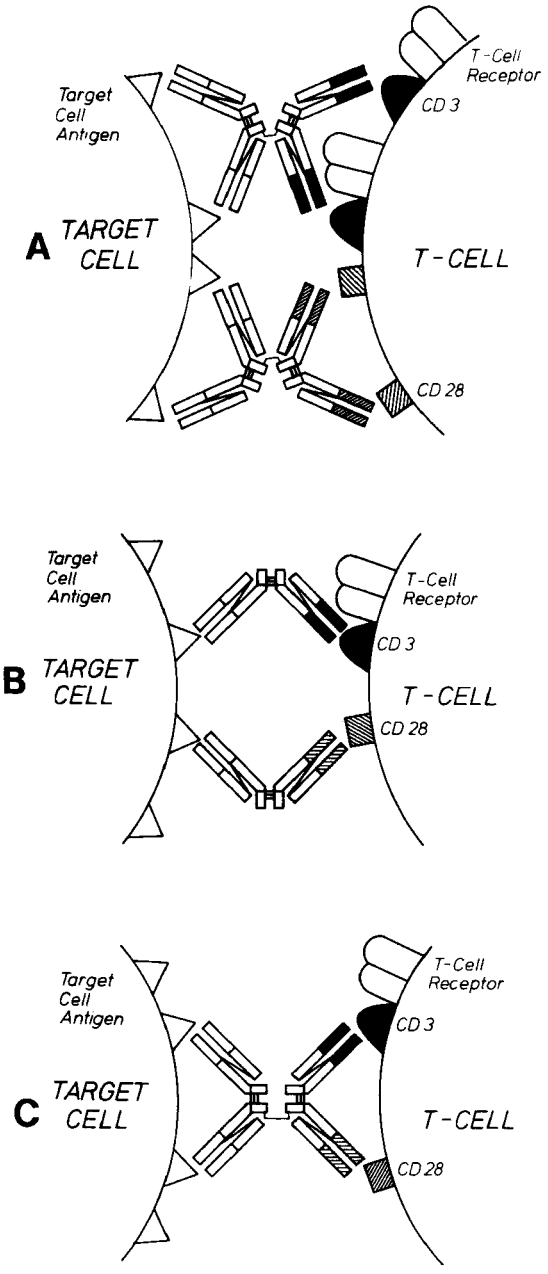

Figure 1. Various types of Fc free bi- and trispecific reagents can "present" on the surface of a target cell the signals necessary for effective activation of resting human T cells namely (a) immobilized anti-CD3 antibody triggering the TcR/CD3 complex and (b) anti-CD28 antibody which provides an accessory signal for T cell activation.

"second" conjugate (9.2.27 × 9.3) or the target cells were omitted from the assay. Similar results were obtained if purified T cells instead of PBMC were used. Taken together the results confirmed previously published observations [7] obtained with conjugates of intact antibodies, in which IL 2R expression and generation of lytic capability were measured to assess T cell activation.

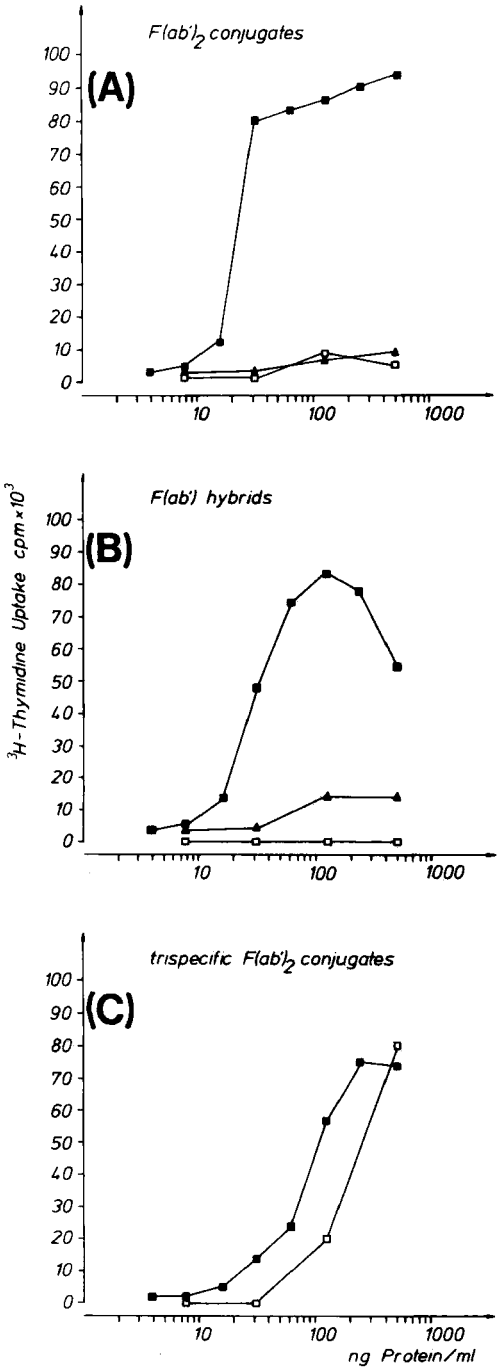

Figure 2. Target cell-induced T cell activation with three types of Fc free bi- and trispecific reagents. (A) Conjugates of $F(ab')_2$ fragments; (B) bispecific Fab hybrids, and (C) a trispecific conjugate of $F(ab')_2$ fragments (see also Fig. 1). PBMC were incubated with: (■) a 1 : 1 mixture of anti-target × anti-CD3 and anti-target × anti-CD28 reagents at the indicated concentrations of each reagent with target cells present, (▲) anti-target × anti-CD3 reagent alone with target cells present, and (□) the anti-target × anti-CD3/anti-target × anti-CD28 combination as above with target cells absent. After 4 days of incubation proliferation was assessed using a [^{3}H]dThd uptake assay.

3.2 Bispecific hybrids of different Fab fragments

To prepare hybrid antibody fragments, univalent for each antigen (Fig. 1B), we modified published procedures [13, 14] as follows (Fig. 3): reduction and subsequent modification of purified $F(ab')_2$ fragments were achieved in one step through reaction (14 h, 25 °C) with a mixture of 10 mM reduced (thionitrobenzoate, TNB) and 10 mM unreduced Ellmans reagent (5,5'-dithiobis-2-nitrobenzoid acid, DTNB; Sigma). This combined reduction and modification step resulted in a substitution rate of 2.5–2.8 TNB residues/Fab fragment without the need to stabilize Fab sulfhydryl groups. After gentle re-reduction of one of the two modified fragments with 0.2 mM DTT (Sigma) for 1 h at 25 °C, 55%–75% of the starting material could be hybridized and separated on a Superdex 200 gel filtration column from unreacted Fab fragments. The spectrophotometric properties of Ellmans reagent allowed easy monitoring of reduction and re-oxidation steps. Using this method we generated three different hybrid reagents with the specificities 9.2.27 × OKT3, 9.2.27 × 9.3 and OKT3 × 9.3.

Fig. 2B shows that a mixture of the anti-target × anti-CD3 and the anti-target × anti-CD28 hybrid, similar to the bivalent conjugates, is capable of mediating target cell-induced T cell activation as defined in this report. However, compared to the bivalent conjugates, we observed notable differences; in all experiments the activity of the hybrid reagents dropped at concentrations above 200 ng/ml of each hybrid. Moreover, with PBMC from 2 out of 15 donors the proliferation observed with the usually effective combination of hybrids was relatively weak (15 000 and 19 000 cpm, respectively, at optimal concentrations of hybrid reagents, data not shown).

That T cell activation as measured by [^{3}H]dThd uptake resulted in tumor cell death is demonstrated in Fig. 4. If mixed cultures of viable tumor cells and PBMC were incubated with the anti-target × anti-CD3 hybrid as a control, tumor cells proliferated vigorously forming an adherent layer at the bottom of the culture flask. After gentle splitting with 0.5 mM EDTA > 90% of these cells were viable in a trypan blue stain. In contrast, if the cell mixture was incubated with an optimal concentration of anti-target × anti-CD3 and anti-target × anti-CD28 hybrids only few adhering cells were observed, forming cell clusters with activated lymphocytes. The fluid phase contained proliferating lymphocytes and few dead tumor cells.

3.3 A trispecific conjugate of $F(ab')_2$ fragments

By chemical coupling of a 9.2.27 $F(ab')_2$ fragment to an OKT3 × 9.3 Fab hybrid using the SPDP cross-linking reagent, we obtained a trispecific $F(ab')_2$ conjugate (Fig. 1C). As expected, this reagent activated T cells in the presence of melanoma target cells (Fig. 2C). However, activation without the target cells was almost equally pronounced, and thus the major portion of the activity of

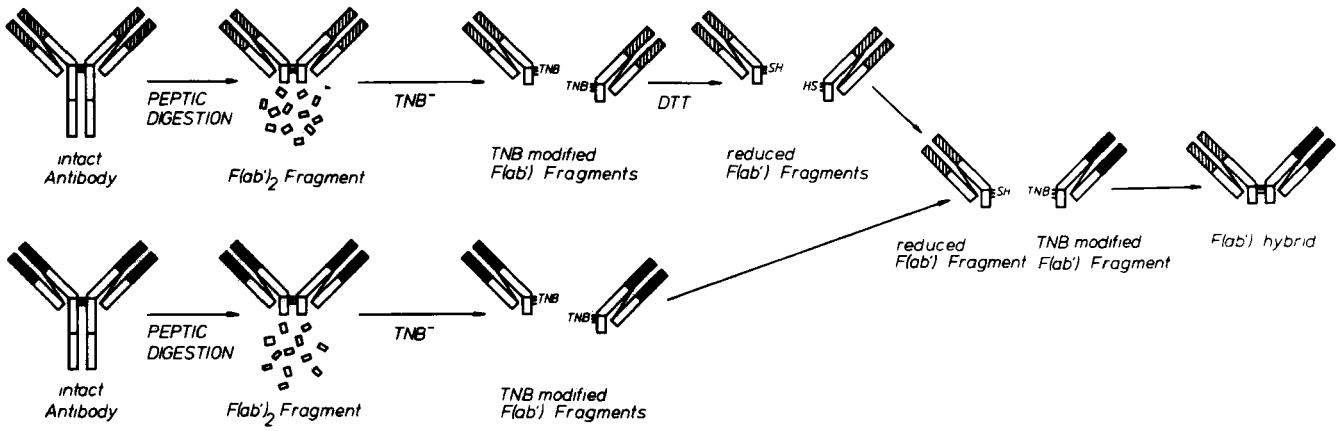

Figure 3. Construction of bispecific Fab hybrids. The major difference from two similar procedures published previously is the reduction of purified $F(ab')_2$ fragments by incubation with a mixture of reduced and unreduced Ellmans reagent. Thus, the TNB anion becomes the reducing agent and reduction and modification of $F(ab')_2$ fragments are achieved in a single step. For details see Sect. 3.2.

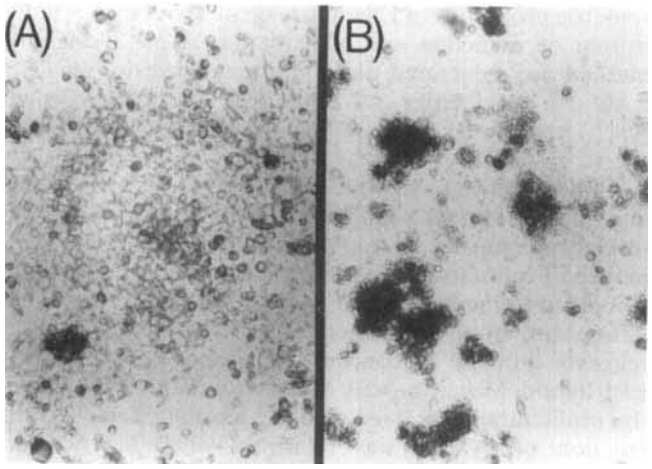

Figure 4. Photomicrographs of viable M21 melanoma cells co-cultured as described in Sect. 2.3 for 4 days with PBMC and (A) the 9.2.27 × OKT3 hybrid, 200 ng/ml or (B) a combination of the 9.2.27 × OKT3 and 9.2.27 × 9.3 hybrid, 100 ng/ml of each reagent. The cell clusters in (B) are activated lymphocytes attached to a few surviving tumor target cells.

this reagent was not target cell induced. We found that this was due to the capability of the soluble OKT3 × 9.3 hybrid to induce proliferation of purified resting T cells (data not shown).

4 Discussion

That bispecific hybrid antibodies, generated by quadroma technology [15, 16], or Fab hybrids [14], as described in this report, are capable of focusing T cell cytotoxicity once it is generated, has been established. However, the use of reagents, that are univalent for each antigen in inducing T cell activation upon binding to a target cell has not been investigated.

In the experiments depicted in Fig. 2, all three types of bi- and trispecific reagents tested induce effective T cell activation in the presence of target cells to which they specifically bind if both the CD3 and the CD28 antigen on the T cell surface become engaged. However, most of the activity of the trispecific conjugate, in contrast to the bispecific reagents, is not target cell dependent (Fig. 2C), since the OKT3 × 9.3 hybrid used to generate this reagent activates T cells in soluble form without its immobilization at the target cell surface being required. This finding was unexpected and its implications for anti-CD3-mediated T cell activation are currently under investigation. In the context of the therapeutic strategy discussed in this report, this result implies that trispecific reagents of the described kind are not suited for systemic *in vivo* application.

Comparing the two types of bispecific reagents, the bivalent conjugates seem to be more effective than the univalent hybrids, in particular when saturating doses, which hamper the required target cell-T cell contact, are used. Since it is well established that the activating properties of anti-CD3 and anti-CD28 antibodies critically depend upon their valency [17, 18], this finding is not unexpected. In addition we found that the affinity of the 9.2.27 × OKT3 hybrid to melanoma target cells is diminished by a factor of five compared to the intact 9.2.27 antibody (data not shown). Despite this possible shortcoming, the advantages of hybrid antibody fragments, such as the absence of foreign chemical groups, their physiological structure and molecular weight, may compensate for the moderate decrease in activity. Moreover, it may be possible to find antibody combinations more effective in T cell activation than the particular anti-CD3/anti-CD28 pair used in our experiments.

In conclusion, it appears possible to generate bispecific reagents which, upon systemic *in vivo* application, induce an *in situ* activation of human T cells on the surface of tumor cells. Whether these reagents are capable of initiating an effective immune response against the target cells *in vivo* remains to be investigated. Nevertheless, we think that the described strategy of a "target cell-induced T cell activation" offers an attractive alternative to concepts, which rely on the *ex vivo* activation of effector cells.

From a theoretical point of view our results underline the efficiency of the CD28 receptor in the delivery of accessory signals for T cell activation. Recently the B cell activation

antigen B7/BB-1 has been identified as a ligand for this receptor [19], and future work may shed light on the physiological role of this accessory pathway.

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Note added in proof: Recently, it was demonstrated in two reports from the same group (Tutt et al., *Eur. J. Immunol.* 1991 *21:* 1351; *J. Immunol.* 1991 *147:* 60) that (a) bispecific antibody fragments with anti-CD3 × antiCD2 specificity induce proliferation of resting PBMC, and (b) a trispecific reagent with anti-target-, anti-CD3- and anti-CD2 specificity is capable of both inducing and redirecting PBMC cytotoxicity. These findings are in accordance with our data, which were obtained using an anti-CD28 antibody as an accessory signal. However, in contrast to Tutt et al., we feel that trispecific reagents are *not* suited for therapeutic application because (a) they induce T cell activation which is *not* target cell dependent, and (b) their bispecific anti-T cell part is likely to mediate mutual T cell killing after activation has occurred.

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