Specific Activation of the Prodrug Mitomycin Phosphate by a Bispecific Anti-CD30/Anti-Alkaline Phosphatase Monoclonal Antibody

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INTRODUCTION

Since the establishment of the hybridoma technique for the production of MAbs3 with defined specificities by Köhler and Milstein (1), many attempts have been made to use MAbs with selective reactivity to cell surface structures on tumor cells as carriers for cytotoxic drugs (2), toxins (3), and radioisotopes (4) in order to deliver high tumoricidal doses to the malignant target cells while minimizing side effects to nonmalignant cells. Despite impressive advances made in in vitro and in vivo models, there remain considerable problems with these immunocomjugates. Due to the limited number of cytotoxic molecules that can be bound to an immunoglobulin without interfering with its antigen-binding capacity, sufficient concentrations of cytotoxic drugs in the tumor are achieved with great difficulty (5). Immunotoxins, such as MAb-racin A chain are more potent, since it has been shown that a single toxin molecule can kill a tumor cell if the toxin reaches its specific site of action (3). This is, however, not possible if the antibody binds to an antigen that is not internalized or is degraded in lysosomes. Moreover, a prerequisite for the effectiveness of immunotoxins for treating tumors is the expression of the immunotoxin-binding antigen on nearly all of the tumor cells, an event that is unlikely to occur as a result of the heterogeneity and instability of the malignant cell population within a given tumor (3). Finally, the modification of antibodies with toxins or cytotoxic drugs by chemical means carries the risk of changing the binding properties of the antibody.

ABSTRACT

The bispecific monoclonal antibody (Bi-MAb) HRS-3/AP-1 was developed by somatic hybridization of the 2 mouse hybridoma cell lines HRS-3 and AP-1, which produce monoclonal antibodies with reactivity against the Hodgkin’s- and Reed-Sternberg cell-associated CD30 antigen and alkaline phosphatase, respectively. After an active incubation with alkaline phosphatase, purified whole immunoglobulin molecules and F(ab’)2 fragments of the Bi-MAb were equally effective in converting a relatively noncytotoxic prodrug, mitomycin phosphate (MOP), into mitomycin alcohol, which was 100 times more toxic to the Hodgkin’s- and Reed-Sternberg cell line L540 (CD30+) than MOP. The cytotoxic activity of MOP was unaffected when the cells were pretreated with either the Bi-MAb or the enzyme alone. The Bi-MAb HRS-3/AP-1 did not bind to HPB-ALL cells (CD30+) and was not able to activate MOP on these cells. In cocultivation experiments with HPB-ALL and L540 cells, the activation of MOP by the Bi-MAb HRS-3/AP-1 and alkaline phosphatase led to considerable cytotoxicity against the antigen-negative bystander cells. Thus, this immunotherapeutic approach might be effective in tumors in which not all the tumor cells express the respective tumor antigen.

MATERIALS AND METHODS

Hybridoma Cell Lines. The production of the parental hybridoma cell lines AP-1 (reactive against bovine alkaline phosphatase) and HRS-3 (reactive against the Hodgkin’s and Reed-Sternberg cell-associated CD30 antigen and only a small subpopulation of normal cells) have been described (9–11). Both antibodies are of the IgG1 subclass.

Development of the Hybrid-Hybridoma Cell Line HRS-3/AP-1. A hypoxanthine-guanine phosphoribosyltransferase-deficient subclone of the hybridoma cell line HRS-3 was obtained by growing this hybridoma in the presence of increasing concentrations of 8-azaguanine (GIBCO, Karlsruhe, Federal Republic of Germany) up to a final concentration of 0.1 mM. AP-1 cells were treated immediately before fusion with 5 mM iodoacetamide in PBS for 5 min on ice according to the method of Clark and Waldmann (12). Hypoxanthine-guanine phosphoribosyltransferase-deficient HRS-3 cells (5 x 10⁶) and iodoacetamide-pre-treated AP-1 cells (1.5 x 10⁶) were mixed and fused using polyethylene glycol as described (13). After fusion, cells were washed extensively, resuspended at a concentration of 10⁶ cells/ml in RPMI-1640 medium containing 10% fetal calf serum and 1% hypoxanthine, aminopterin, and thymidine (GIBCO) and seeded into the wells of a 96-well microtiter plate (Nunc, Wiesbaden, Federal Republic of Germany). Two weeks after plating, supernatants from wells with growing clones were tested for the presence of Bi-MAbs using a modified form of the previously described APAAP (14). In short, 5 µl of a suspension of the Hodgkin’s-derived cell line L540 (10⁶ cells/ml in RPMI 1640 + FCS) were seeded into the wells of a 60-well Terasaki plate (Nunc) and fixed with 0.1% (v/v) glutaraldehyde in PBS. Five µl of the supernatants were incubated for 30 min. After washing, cells were incubated with AP (0.1 mg/ml) in PBS for 30 min. After repeated washing, the reaction was developed by incubation with 0.2 mg/ml naphthol-AS-BI-phosphate and 1 mg/ml Fast Red TR salt (5-chloro-2-toluenediazonium...
chloro-hemi-zinc chloride; Sigma, Munich, Federal Republic of Germany) in Tris/HCl (pH 8.2) until a red reaction product developed. The hybrid hybridoma cell line producing the supernatant with the strongest reactivity in this modified APAAP was designated HRS-3/AP-1 and subcloned repeatedly by limiting dilution until a stable subclone was obtained.

**Purification and Characterization of the Bi-MAb HRS-3/AP-1.** Antibodies were produced in BALB/c mice by injection of 0.5-3 x 10^6 hybrid hybridoma cells. An N-hydroxysuccinimide-agarose column (Affi-Gel 15; Pharmacia, Freiburg, Federal Republic of Germany) was coated with calf intestinal AP (Sigma) according to the manufacturer's instructions. For immunoadsorption of HRS-3/AP-1, undiluted culture supernatants or a 1:10 dilution of ascites in PBS was processed over the AP/Affi-Gel 15 column and the bound fraction was diluted with a 0.1 M sodium phosphate buffer gradient ranging from pH 7.2 to pH 3.0. The protein concentration of the eluate was monitored by determining the absorption at 280 nm. Aliquots of the eluate were tested for the presence of Bi-MAb as described above. Bi-MAb-containing samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions in 10% slab gels according to the method of Laemmli (15). Protein staining was performed using Coomassie brilliant blue (Pharmacia).

**Preparation of F(ab')2 Fragments.** F(ab')2 fragments of HRS-3/AP-1 were prepared according to the method of Lamoyi (16). The purified Bi-MAbs were dialyzed against 0.1 M sodium acetate buffer (pH 7.0) at 4°C overnight. Immediately before digestion, the pH of the protein solution was adjusted to 4.2 by adding acetic acid, and incubated at 37°C with a 3:100 (w/w) pepsin:immunoglobulin ratio in 0.1 M acetate buffer, pH 4.2. The reaction was stopped after 4 h by adding 0.5 N NaOH to a final pH of 8.0. The solution was then dialyzed against PBS. F(ab')2 fragments were purified on a protein A-Sepharose column that was equilibrated and washed with 1.5 M glycine; 3 M NaCl, pH 8.9; followed by 0.1 M citrate, pH 3.0. F(ab')2 fragments were collected in the flow-through and undigested immunoglobulin was found in the acid eluate.

**Prodrugs and Cytotoxic Drugs.** MMC was obtained from Medac (Hamburg, Federal Republic of Germany). MOP was synthesized from mitomycin A and 2-aminoethyldihydrogen phosphate as described previously (8).

**Target Cells.** The CD30+ Hodgkin’s-derived cell line L540 (17) and the CD30- cell line HPB-ALL, which is derived from a human acute T-lymphocytic leukemia, were used for cytotoxic assays. All cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum (GIBCO).

**Trypan Blue Dye Exclusion Assay.** Target cells (4 x 10^5/ml) were pretreated at 37°C for 30 min with the Bi-MAb HRS-3/AP-1 (10 µg/ml). After washing, cells were incubated with AP (0.1 mg/ml) for 30 min. Untreated cells and cells pretreated with the Bi-MAb HRS-3/AP-1 alone or AP alone were used as controls. One hundred µl of the cell suspension (4 x 10^5/ml in RPMI 1640 + 10% FCS) were seeded into the wells of a 96-well microtiter plate, followed by incubation with various concentrations of MMC or MOP (ranging from 0.1 µM to 1 mM) for 24 h at 37°C, 5% CO2. Cells were harvested, mixed with trypan blue solution, and counted. All experiments were performed in quadruplicate and repeated at least three times.

**Chromium Release Assay.** The chromium release assay was performed according to the method of Sanderson (18) with the following modifications. Cells were labeled with 51Cr (100 µCi/10^6 cells for 1 h) and washed three times. Labeled cells were pretreated as described for the dye exclusion test. One hundred µl of a suspension of labeled pretreated and control cells (10^4 cells/ml in RPMI 1640 + 10% FCS) were seeded into the wells of 96-well microtiter plate. MMC (10 µM), MOP (10 µM), or medium was added for 18 h at 37°C at 5% CO2. The supernatants of each well were harvested and assayed for radioactivity. The percentage of cytotoxicity was calculated as:

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100 \times \frac{\text{Counts experimental release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}}
\]

Maximal release of 51Cr was defined as the release obtained by the addition of Nonidet P-40 (Sigma, Munich) to a final concentration of 2% (v/v).

**Cold Target Assay.** To determine the effect of MOP activation on antigen-negative cells, unlabeled L540 (10^4 cells/ml) and 51Cr-labeled HPB-ALL cells (10^4 cells/ml) were pretreated as described previously, mixed at a ratio of 1:1, and incubated with MOP, MMC, or medium as described above.

**RESULTS**

Production of Bispecific Monoclonal Antibody. After fusion of 5 x 10^6 HRS-3 cells with 1.5 x 10^7 AP-1 cells, 28 clones grew out in the selective medium. The supernatants of 26 clones were positive in the screening assay using the modified APAAP method (see “Materials and Methods”) indicating the production of Bi-MAbs with reactivity against both CD30 and AP. The clone that produced the supernatant with the highest reactivity was selected for repeated subcloning and established as Bi-MAb HRS-3/AP-1. Ascites containing the Bi-MAb was precipitated by ammonium sulfate and fractionated over an AP column. Of the different antibody combinations produced by the hybrid hybridoma cell line HRS-3/AP-1, MAbs with bi-valence (monospecific MAbs) and mono-valence (i.e., bispecific MAbs, the second valence being directed against the CD30 antigen) for AP were bound to the column, while the antibodies without reactivity to AP (i.e., monospecific anti-CD30 MAbs) were found in the flowthrough. The elution curve of the AP-column showed 2 peaks, 1 at pH 5.3 and 1 at pH 3.4. The latter is identical to the peak obtained with purified AP-1 (Fig. 1). The peak at pH 5.3 contained the Bi-MAb HRS-3/AP-1 as demonstrated by reactivity in the modified APAAP on L540 cells, while the second peak contained MAbs with reactivity to AP only.

The purity of the antibody preparations was then analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2). The monospecific HRS-3 and AP-1 MAbs can be distinguished by their different light chains, whereas their heavy chains cannot be distinguished electrophoretically. The peak at pH 5.3 contained 2 light chains that represented the light chains of each fusion partner in the Bi-MAb HRS-3/AP-1.

Trypan Blue Dye Exclusion Assay. Over a wide range of concentrations, MMC proved to be about 100-fold more toxic to the Hodgkin’s-derived L540 cells than MOP (Fig. 3). When the Hodgkin’s cells were incubated with MOP after pretreatment with both the Bi-MAb HRS-3/AP-1 and AP, the cytotoxicity of MOP increased to the same level as MMC. In contrast, pretreatment by either the monospecific antibody HRS-3, the Bi-MAb HRS-3/AP-1, or AP alone did not alter the cytotoxic potency of MOP.

Similar to the Hodgkin’s-derived cell line L540, the human acute T-lymphocytic leukemia cell line HPB-ALL proved to be 100 times more sensitive to the cytotoxic activity of MMC compared with the prodrug MOP. However, in contrast to the CD30+ cell line L540, pretreatment of the CD30- HPB-ALL cells with Bi-MAb and AP did not increase the cytotoxic activity of MOP (Fig. 3).

51Cr Release Assay. The drugs and prodrugs were tested at 10 µM based on the reproducible levels of significant cytotoxic activity of both MMC and the released drug mitomycin alcohol (the active derivate of MOP) and the minimal cytotoxicity of the prodrug MOP at this concentration (Figs. 3 and 4). The amount of 51Cr release for MMC was significantly higher (91% from L540, 82% from HPB-ALL) than for the prodrug MOP (1% from each cell line; Fig. 4). Again, Bi-MAb HRS-3/AP-1
alone was unable to increase the toxicity of the prodrug, whereas pretreatment with whole immunoglobulin molecules of the Bi-MAb and AP led to a cytotoxic activity against L540 that was comparable with that of MMC (73%). Pretreatment of the CD30 antigen-negative HPB-ALL did not alter the toxic activity of the prodrug. The F(ab')2 fragments of the Bi-MAb HRS-3/AP-1 were as effective as whole immunoglobulin molecules in conferring prodrug activation after binding of AP (Fig. 4).

Effect of Specific Prodrug Activation on Antigen-negative Bystander Cells. To test whether the activation of the prodrug MOP by the Bi-MAb and AP on CD30+ cells affects antigen-negative cells in the neighborhood of these cells, both unlabeled CD30+ L540 and 51Cr-labeled CD30- HPB-ALL cells were treated with Bi-MAb and AP and were mixed and incubated with the prodrug and the relevant controls (Fig. 5). Again, MMC proved to be quite toxic to HPB-ALL. However, in contrast to the results obtained when HPB-ALL cells were incubated alone, the release of 51Cr from HPB-ALL cells in the presence of L540 cells pretreated with Bi-MAb and AP increased significantly, even though the values of the release induced by the active compound MMC were not quite reached. Incubation of nonpretreated unlabeled L540 cells together with Bi-MAb/AP-pretreated HPB-ALL cells did not increase the cytotoxicity of MOP (Fig. 5).

DISCUSSION

Hybrid hybridoma cells produce the 2 parental light and heavy chains, which are recombined by chance, so that as many as 10 different immunoglobin molecules are secreted into the supernatant (19). Purification of the Bi-MAb is mandatory in order to separate the monospecific antibodies that can block the functions of the Bi-MAbs. In the case of the Bi-MAb with reactivity to the Hodgkin’s-associated CD30 antigen and AP, purification was achieved using a column coated with AP. Since the monospecific antibodies with reactivity to AP have a higher affinity to the antigen than do the Bi-MAbs, they can be eluted separately providing a highly purified preparation of Bi-MAb.

Using the purified Bi-MAb HRS-3/AP-1 with reactivity to the Hodgkin’s-associated CD30 antigen and alkaline phosphatase, we were able to demonstrate that the relatively nontoxic prodrug MOP can be activated specifically on antigen-positive cells. The released drug mitomycin alcohol proved to be as toxic as the active compound MMC on a molar basis. We were able to demonstrate that the prodrug is specifically activated on the surface of the antigen-positive cell to which the enzyme is bound via the Bi-MAb. The fact that F(ab')2 fragments were as effective as whole immunoglobulin molecules excludes the possibility that the binding of the BiMAb is mediated by Fc receptors.

One advantage that antibody-enzyme complexes have over direct antibody-drug conjugates is that antigen-negative tumor cells in the neighborhood of antigen-positive cells can also be reached by the released drug. That this is indeed the case was demonstrated by the results of cocultivation experiments of the CD30+ L540 and the CD30- HPB-ALL cells: the released drug, which is specifically generated on pretreated antigen-positive Hodgkin’s cells, can also gain access to antigen-negative cells and exert cytotoxic activity. Thus, this immunotherapeutic approach might be effective in vivo, where not all the tumor cells carry the respective tumor antigen. It is expected that this...
approach will lead to some normal cell destruction, but hope-fully less than that which would occur through systemic drug administration, since a large portion of the active drug will be released at the target site. The observation that the cytotoxic activity is weaker on antigen-negative bystander cells than on antigen-positive cells suggests that a considerable proportion of the active compounds are directly internalized into the Bi-Mab-enzyme complex-bearing cell. Therefore, most of the toxic effects to normal cells should be confined to cells located in the neighborhood of tumor cells.

Our results extend previous observations on the enhancement of antitumor activity of phosphorylated prodrugs such as MOP and etoposide phosphate on colorectal and lung cancers in vitro and in vivo (6, 8). In these previous studies, immunoconjugates were produced by covalently binding alkaline phosphatase to an antitumor antibody via a thioether bridge. Immunoconjugates formed in this manner have several theoretical and practical disadvantages when compared with Bi-MAbs; the process of the chemical coupling of a MAb alters its affinity, and the stability of the conjugate is often not satisfactory. In contrast, the use of Bi-MAbs [whole immunoglobulin molecules and F(ab')2 fragments] provides a stable product with consistent binding properties and uniform composition. Particularly F(ab')2 fragments may prove to be valuable for therapeutic applications in vivo because of their shorter half-time in plasma, better tumor penetration, and the lack of unspecific binding via
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Fc receptors. In addition, when using antibodies against the AP type of the tumor-bearing host, the Bi-MAb may be able to localize endogenous phosphatases present in the body, thereby reducing prodrug hydrolysis at nontarget sites.

In summary, our results obtained with Bi-MAbs against a Hodgkin's-associated antigen and AP may serve as a model for many tumors for which MAbs with restricted reactivity to tumor cells are available. The AP-mediated activation of cytotoxic prodrugs on tumor cells is not restricted to phosphates of etoposide or mitomycin since other hydroxyl-containing cytotoxic drugs may be amenable to phosphorylation, thus opening the possibility of polychemoimmunotherapy. Because of its restricted reactivity to Hodgkin's and Reed-Sternberg cells, the malignant cells of certain lymphomas and only a small subpopulation of normal cells (13), HRS-3 might be a good candidate for immunodiagnostics and immunotherapy in patients. Indeed, we recently demonstrated that HRS-3 binds to the malignant Hodgkin's and Reed-Sternberg cells of patients with Hodgkin's disease in vivo (20, 21). Studies using less immunogenic (“humanized”) Bi-MAbs with reactivity against human AP will be the subject of future investigations to show whether this approach opens new avenues in the treatment of human cancer.

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