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Preparation and analysis of bifunctional immunoconjugates containing monoclonal antibodies OKT3 and BABR1

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Summary. OKT3 and BABR1 [anti-(breast tumour) antibody] were conjugated using *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). The procedure employed mild reducing conditions for SPDP-BABR1 and short conjugation incubations at 37° C. The bifunctional immunoconjugates thus produced were isolated by HPLC gel filtration on a preparative TSK 3000 SW column. Both intact IgG and $F(ab')_2$ fragments have been conjugated. The ratio of SPDP to IgG for the optimal yield of dimeric OKT3-BABR1 heteroconjugates was determined to be 2 for OKT3 and 1–2 for BABR1. The OKT3-BABR1 dimers were shown to be bifunctional heteroconjugates by polyacrylamide gel electrophoresis, isoelectric focusing, radial immunodiffusion, and flow cytometry. The binding specificities of the bifunctional heteroconjugates were identical to the specificities of both parent antibodies.

Introduction

The cytolytic activity of effector T cells is enhanced in the presence of bifunctional antibody heteroconjugates, which bind both to the T cell receptor complex and to a target cell-surface antigen [1, 10, 13, 15, 17, 19, 23]. Effector cells that exhibit this enhancement include murine [1] and human [13, 17] cytotoxic T lymphocytes and human peripheral blood T cells [18]. The enhancement in cytolytic activity is much greater following treatment of the effectors with recombinant human interleukin-2 (rIL-2) [15, 18]. Both fresh and cultured human tumour cells are efficiently lysed by effectors in the presence of heteroconjugate [18]. Furthermore, human peripheral blood T cells treated with heteroconjugate prevented the growth of tumour cells in nude mice [28, 29]. These results suggest that heteroconjugates may be clinically useful in conjunction with rIL-2-activated cells [14, 15], which have already been shown to be therapeutically beneficial [21, 29].

Bifunctional heteroconjugates are prepared by covalently joining two purified antibodies using a heterobifunctional cross-linking agent such as *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). The resulting conjugates are separable from unconjugated IgG by gel filtration chromatography. In this study we describe modifications of this method that optimize the purity, chemical integrity, and yield of dimeric heteroconjugates containing OKT3 and the anti-(breast tumour) antibody BABR1.

Materials and methods

Reagents. Caprylic acid, sodium azide, and TRIS were obtained from Sigma. Ultra-pure-grade dithiothreitol was purchased from Boehringer-Mannheim. Iodoacetamide was obtained from Kodak. Acrylamide, *N,N'*-methylenebisacrylamide, riboflavin 5'-phosphate, and *N,N,N',N'*-tetramethylenediamine were obtained from Bio-Rad. Ampholytes were purchased from Pharmacia, SPDP was obtained from Pierce Chemicals.

Antibodies. Hybridomas secreting OKT3 and the anti-(breast tumour) antibody BABR1 were obtained from the American Type Culture Collection. Antibodies were purified from ascites by caprylic acid precipitation [24] and subsequently by affinity chromatography with protein-A-Sepharose CL-4B (Pharmacia) as described [8] or with protein-A-agarose (Pierce Chemicals).

$F(ab')_2$ preparation. Purified IgG was dialyzed against 0.1 M sodium acetate, pH 7.0, and then concentrated to 20 mg/ml in 0.1 M sodium acetate, pH 4.2, by using a Centricon-30 concentrator. A 10 mg/ml stock solution of pepsin (Worthington) was prepared fresh and added to the IgG in a final weight ratio of 5% pepsin to IgG. The reaction mixture was incubated at 37° C for 18 h for IgG1 or 12 h for IgG2a. The reaction was terminated by adjusting the pH to 7.0 by the addition of 0.1 M NaOH. $F(ab')_2$ fragments were purified by HPLC gel filtration on TSKR 3000 SW as described below and subsequently by affinity chromatography using protein-A-Sepharose CL-4B as described [8].

SPDP conjugation. Bifunctional heteroconjugates and homoconjugates of antibodies were prepared using SPDP by a modification of a published procedure [5]. Each mole of antibody was incubated with 1–3 moles of SPDP for 30 min at room temperature in Dulbecco's modified [7] phosphate-buffered saline without $CaCl_2$ and $MgSO_4$ (PBS, Mediatech). SPDP (20 mM in ethanol) was added to the antibody dropwise with vigorous stirring in Reactivials (Pierce Chemicals). Derivatized antibodies were iso-

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lated by gel filtration on Sephadex G-25 (Pharmacia). One derivatized antibody was reduced by incubation with 450 μM dithiothreitol in 0.1 M sodium acetate, 0.1 M NaCl (pH 4.5) for 30 min at room temperature. Reduction was monitored spectrophotometrically as the release of pyridine-2-thione, which absorbs at 343 nm. Thus the extent of SPDP derivatization was calculated from its molar absorption coefficient of 8.08×10^3 $M^{-1}cm^{-1}$ [26]. The reduced antibody derivative was isolated by gel filtration on a Sephadex G-25 column equilibrated in PBS containing 1 mM EDTA. Antibodies were concentrated to 10 mg/ml by centrifugation in an Amicon Centricon-10. The reduced and nonreduced antibody derivatives were incubated in equimolar amounts at a concentration of 10 mg/ml in PBS containing 1 mM EDTA at 37° C for 1 h.

HPLC gel filtration. Heteroconjugates were isolated by HPLC gel filtration on a TSK 3000 SW column (21.5 × 600 mm, Beckman Instruments Inc.) equilibrated with 0.1 M sodium phosphate, 1 mM EDTA (pH 6.8) at a flow rate of 2.0 ml/min. The absorbance of the column effluent was measured at 280 nm, and fractions were collected every minute. Gel filtration calibration standards (13.7–2000 kDa) were obtained from Pharmacia.

SDS polyacrylamide gel electrophoresis. Samples were analyzed by SDS gel electrophoresis under both reducing and nonreducing conditions. Reducing gels were 5%–15% gradient sodium dodecyl sulfate (SDS)/polyacrylamide gels with 3% stacking gels, and the Laemmli buffer system was used [12]. Samples were reduced by incubation in sample buffer containing 5% (v/v) 2-mercaptoethanol at 100° C for 5 min. Nonreducing gels were 2%–16% gradient polyacrylamide slab gels run in the absence of 2-mercaptoethanol. Samples consisted of 10 μg protein, and electrophoresis proceeded at 11 mA for 16 h for both gel procedures. The gels were stained with Coomassie brilliant blue. Molecular mass standards (14.3–200 kDa) were obtained from Bethesda Research Laboratories.

Protein determination. Protein concentrations were determined by using the method of Bradford [2].

Isoelectric focusing. Antibodies and heteroconjugates were reduced and alkylated by incubation for 30 min at room temperature in 1 M TRIS/HCl, 0.1 M dithiothreitol, 1 mM EDTA (pH 8.0). Crystalline iodoacetamide was added to a concentration of 0.25 M with vigorous stirring. The mixture was incubated for 30 min at room temperature in the dark and then desalted by passage through a Sephadex G-25 column equilibrated in PBS. Reduced and alkylated antibodies were concentrated to 1 mg/ml in a Centricon-10 apparatus (Amicon). The 5% polyacrylamide gels used for focusing contained 13.3% glycerol, 1% Triton X-100, 0.5% riboflavin 5'-phosphate, and 5% ampholine pH 3.5–10. Gels were prefocused for 1 h at 8° C at a constant power of 30 W. For silver-stained gels 10–15 μg protein was used for samples, whereas 20–30 μg protein was used for Coomassie-blue-stained gels. Samples were focused for 2–3 h at 8° C at 1430 V. The gels were fixed in 10% trichloroacetic acid, 4% sulfosalicylic acid for 30 min, washed in water for 1 h, and stained with silver by the method of Morrissey [16] or with Coomassie brilliant blue R-250, 0.04% in 27% isopropanol, 10% acetic acid, 0.5% $CuSO_4$.

Single radial immunodiffusion in agar gels. The method of Clausen [6] was used. Prepared immunodiffusion plates containing mouse antibody to IgG1 or IgG2a, mouse immunoglobulin standard proteins, and sheep serum diluent were in IgG1 and IgG2a single radial immunodiffusion kits obtained from Miles Scientific. Standard samples of high, medium and low concentration, and heteroconjugate were applied to wells of IgG1 and IgG2a plates. After 4 days at room temperature the sizes of the precipitin rings were measured, standard curves constructed, and the concentration of IgG1 or IgG2a in heteroconjugate determined from the standard curves.

Lymphokine-activated effector cells. Mononuclear cells from a normal donor or from patients with advanced malignancies were used as $CD3^+$ targets to measure antibody binding by flow cytometry. Mononuclear cells at 3×10^6/ml were activated in culture with recombinant human interleukin-2 (rIL-2, Cetus Corporation) at 1000 U/ml in RPMI culture medium with 2% human AB serum, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 mg/ml gentamicin sulfate (all from M. A. Bioproducts Inc.). Cells were fresh or were resuspended at 1×10^7/ml in freezing medium containing 40% RPMI, 10% dimethyl sulfoxide (Sigma), and 50% fetal bovine serum (FBS, Hyclone Labs). Frozen cells were thawed rapidly at 37° C and washed twice in RPMI with 5% FBS. Surface $CD3^+$ cells ranged from 75% to 90% in different effector populations.

Tumour cell lines. Human tumour cell lines, obtained from the American Type Culture Collection, include BT-20 (breast carcinoma), BT-474 (breast ductal carcinoma), MCF-7 breast adenocarcinoma), ZR-75-1 (breast carcinoma), WiDr (colon adenocarcinoma), and SK-Mel-1 (melanoma). Melanoma line CaCL 78-4 was developed and provided by Dr. S. K. Liao, Biotherapeutics Inc., Franklin, Tenn. All tumour lines were maintained in log growth in tumour culture medium containing RPMI 1640 with 2 mM L-glutamine, 1 mM sodium pyruvate solution, 15 mM HEPES, 20 μg/ml gentamicin (all from M. A. Bioproducts Inc.), and 15% FBS. Adherent cell lines were trypsinized (10 min at 37° C) to obtain cell suspensions. Cells were washed twice in RPMI medium containing 5% FBS, resuspended in freezing medium at 5×10^6/ml, and frozen in 1-ml aliquots. Frozen cells were thawed rapidly at 37° C and washed twice with RPMI containing 5% FBS.

Immunofluorescent staining. For indirect staining, washed effectors or targets at 1×10^6 cells/test were resuspended in approximately 20 μl wash/dilution buffer (PBS, 2% FBS, 0.1% sodium azide) and were incubated in 200 μl 10 μg/ml T3BR1 heteroconjugate (containing OKT3 and BABR1), OKT3, BABR1, or nonspecific mouse immunoglobulin for 30 min at 4° C. After washing twice, cells were resuspended in 20 μl media as before and incubated for 30 min at 4° C with 200 μl goat anti-(mouse IgG) labeled with fluorescein isothiocyanate (Coulter Immunology) at the recommended dilution. Cells were again washed twice and resuspended in a final volume of 500 μl 2% paraformaldehyde in PBS.

Flow cytometry. Flow cytometry was performed on an Epics Profile flow cytometer (Coulter Electronics)

equipped with a 40-mW air-cooled argon ion laser (Omnichrome) emitting 488 nm at 15 mW. Green fluorescence (fluorescein isothiocyanate) was collected through a combination of 515-nm long-pass absorbance and a 530-nm short-pass filter. Calibration was done daily using fluorescent beads (Coulter Immuno-check Alignment Fluorespheres) to obtain a coefficient of variation for forward light scatter of less than 2.0%. Sheath pressure was 7.5 lb/in^2 (51.7 kPa), and the sample flow rate was 300–500 cells/s. Data analysis was performed on Epics Profile software version 1.2.

Results

SPDP derivatization of IgG

Purified BABR1 and OKT3 were derivatized with a twofold molar excess of SPDP. Complete reduction of these 2-pyridyl disulfide derivatives was obtained by the addition of 25 mM dithiothreitol to aliquots of both derivatives. After a 30-min incubation at room temperature, the absorbances were measured at 343 nm. Using the molar absorption coefficient of $8.08 \times 10^3\ M^{-1}cm^{-1}$ [26] of pyridine-2-thione, the molar ratios of 2-pyridine disulfide to IgG were calculated to be 1.91 for BABR1 and 1.92 for OKT3 for a typical derivatization (95% efficiency).

Reduction of 2-pyridyl disulfide IgG

The reduction of the 2-pyridyl disulfide BABR1 derivative (188 nmol in 3.0 ml) was examined by the addition of increasing concentrations of dithiothreitol. The reaction was performed in 0.1 M sodium acetate, 0.1 M NaCl (pH 4.5). Reduction was monitored spectrophotometrically by absorbance at 343 nm. The reaction proceeded at room temperature for 5 min between additions of dithiothreitol aliquots. The results are shown in Fig. 1. The absorbance of the reaction mixture increased linearly with increasing dithiothreitol concentration up to 100 μM dithiothreitol. The A_{343} reached a plateau value at 160 μM dithiothreitol, consistent with essentially complete reduction of the pyridine disulfide structures.

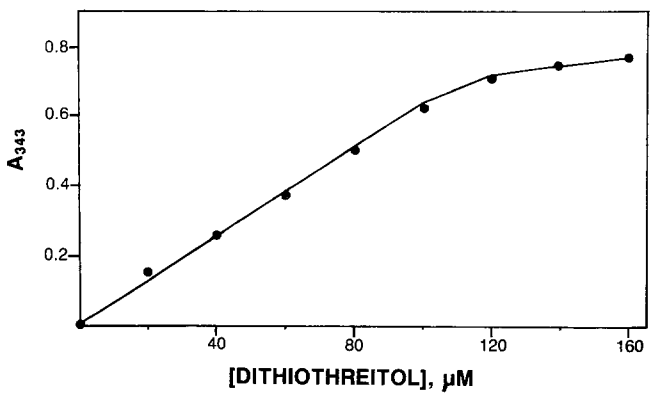

Fig. 1. Reduction of the BABR1 SPDP derivative with dithiothreitol. BABR1 (30 mg) was derivatized with a threefold molar excess of SPDP and dialyzed against 0.1 M sodium acetate (pH 4.5). Aliquots (10 μl) of 1 mM dithiothreitol were added at 5-min intervals and the absorbance was measured at 343 nm

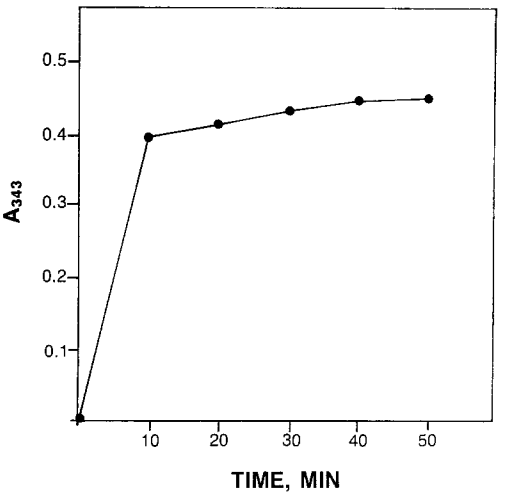

Fig. 2. Conjugation of BABR1 and OKT3. Conjugation of BABR1 and OKT3 SPDP derivatives was monitored by absorbance of pyridine-2-thione at 343 nm. The reaction mixture was incubated at 37° C

Conjugation of BABR1 and OKT3

BABR1 and OKT3 were derivatized with a twofold molar excess of SPDP. The BABR1 derivative was reduced by the addition of 450 μM dithiothreitol and isolated by passage through Sephadex G-25. Reduced BABR1 and nonreduced OKT3 were conjugated by incubation at 37° C. The extent of conjugation was determined spectrophotometrically by monitoring the elimination of pyridine-2-thione, which absorbs at 343 nm. The A_{343} was measured at 10-min intervals as shown in Fig. 2. Most of the absorbance increase occurred in the first 10 min of the reaction. The reaction was essentially complete at 50 min since no further increase in A_{343} was observed.

Isolation of heteroconjugates by HPLC gel filtration

BABR1 (6.3 mg) and OKT3 (5.7 mg) were derivatized using a threefold molar excess of SPDP and conjugated. The resulting preparation was chromatographed on TSK 3000 SW as described above. The results are shown in Fig. 3. Three peaks eluted from the column. The first peak coincided with the void volume with a retention time of 40 min. High-molecular-mass conjugates, consisting of five or more IgG molecules, were expected to elute at this retention time since IgM (900 kDa) was observed in the void volume fraction (see inset). Monomeric IgG eluted with a retention time of 55 min as expected (see inset). Another peak eluted with a retention time of 46 min, which was approximately the retention time expected of a dimeric heteroconjugate (320 kDa). This peak was well resolved from the monomer and mostly resolved from heteroconjugates of higher molecular mass. The elution of molecular mass standards from preparative HPLC TSK 3000 SW was determined, and their retention times were plotted as a logarithmic function of their molecular masses as shown in the inset. Resolution of these proteins was optimal within the molecular mass range of 43–440 kDa. Monomeric murine IgG (160 kDa) eluted at 54 min and is shown in the inset by an open circle. The elution of dimeric and trimeric heteroconjugates is also shown by open cir-

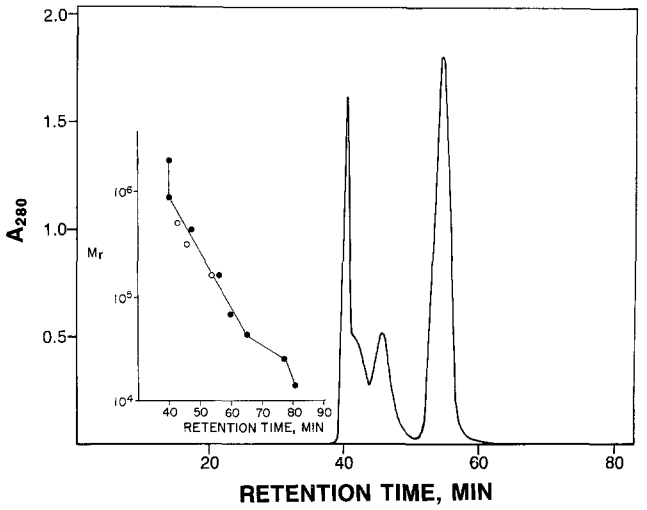

Fig. 3. HPLC gel filtration of T3BR1 heteroconjugate preparation. BABR1 and OKT3 were both derivatized with a threefold molar excess of SPDP and conjugated as described in the text. The conjugation reaction mixture was chromatographed on a TSK 3000 SW column. The peak at 54 min retention time is IgG monomer. Calibration standards (13.7–2000 kDa) were also run on this TSK 3000 SW column. Elution was monitored at 280 nm. The retention times of the standards were plotted as a logarithmic function of their molecular mass as shown in the *inset* (●). The elution of IgG monomer, dimer, and trimer were plotted (○)

cles. Trimeric heteroconjugate (retention time = 42 min) is clearly seen in conjugations using a twofold molar ratio of SPDP to IgG (data not shown).

SDS/polyacrylamide gel electrophoresis

The peak fractions from Fig. 3 were analyzed by both nonreducing and reducing polyacrylamide gel electrophoresis (Fig. 4). The center panel shows the nonreducing gel for the HPLC peak fractions 41, 46, and 55 in lanes 1, 2, and 3 respectively. Fraction 41, corresponding to the void volume of the column, contained high-molecular-mass protein migrating diffusely at the top of the gel. Fraction 46 contained a band of protein with a migration expected for dimeric IgG. Fraction 55 contained monomeric IgG and free heavy and light chains. These results together with the gel filtration data confirm that IgG monomer eluted from the column at 55 min, dimeric heteroconjugate eluted at 46 min, and oligomeric heteroconjugate eluted with the void volume at 41 min. The right panel of Fig. 4 shows the reducing gel of fractions 41, 46, and 55. All the protein in these fractions migrated as free heavy and light chains. The IgG2a heavy chain of OKT3 migrated slightly more slowly than the IgG1 heavy chain of BABR1, as confirmed by running the two purified antibodies side-by-side on reducing polyacrylamide gels (data not shown). Thus lane 2 shows that fraction 46 contained equal amounts of the two antibodies as expected for a dimeric heteroconjugate.

Effects of varying the ratio of SPDP to IgG

Various molar ratios of SPDP to IgG were investigated in order to optimize the yield of dimeric heteroconjugate. With a twofold molar ratio of SPDP to IgG the yield of conjugates was the same as for a threefold molar excess SPDP (Fig. 3), but a much greater fraction of the reaction products were dimers (Table 1). There was a peak at 43 min, slightly resolved from the high-molecular-mass conjugate peak at 40 min. Samples of each peak fraction were analyzed by nonreducing electrophoresis (data not shown). Fraction 43 contained a diffuse band migrating more slowly than the dimeric heteroconjugate, which is interpreted to be trimeric IgG on the basis of both gel filtration and electrophoretic data. In another conjugation experiment BABR1 was derivatized with a threefold molar excess of SPDP, and OKT3 was derivatized with equimolar SPDP in order to limit the formation of high-molecu-

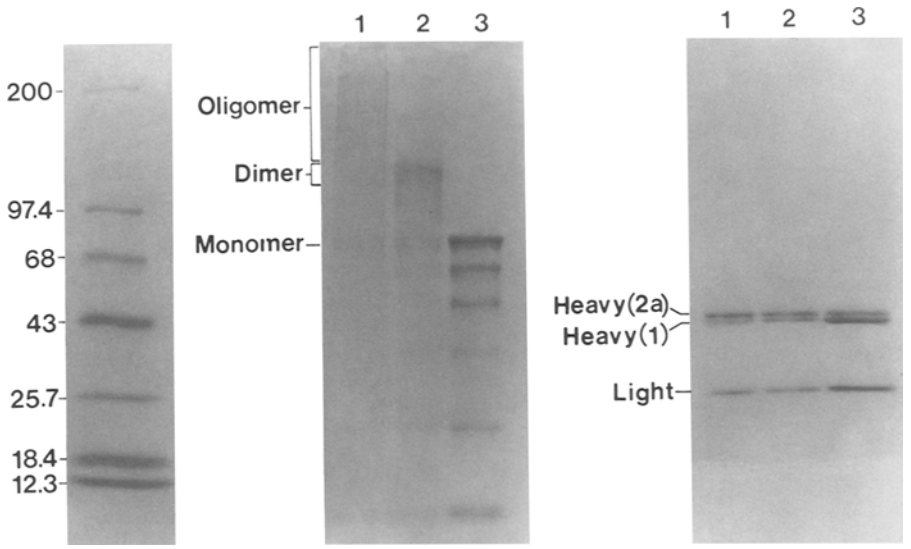

Fig. 4. SDS/polyacrylamide gel electrophoresis of T3BR1 fractions. The peak fractions of heteroconjugate preparation T3BR1 shown in Fig. 3 were analyzed by nonreducing electrophoresis (*center panel*) and reducing electrophoresis (*right panel*). Molecular mass markers for the nonreducing gel are shown (*left panel*). The peak fractions run in lanes *1, 2,* and *3* contained primarily IgG oligomer, dimer, and monomer respectively. All the protein in the peak fractions was reduced to free heavy chains and light chains by 2-mercaptoethanol as shown in the *right panel.* The heavy chains for OKT3 (IgG2a) and BABR1 (IgG1) were resolved on this gel system

Table 1. Effect of varying SPDP/IgG molar ratios upon the relative abundance of the conjugation reaction products

Molar ratio of SPDP/IgG[a]		Reaction product composition[b] (%)		
OKT3	BABR1	Trimer + oligomer	Dimer	Monomer
3.0	3.0	42.2	8.5	49.2
2.0	2.0	24.0	25.8	50.1
1.0	3.0	11.2	15.2	73.6
2.0	1.0	6.9	24.2	68.9

[a] The molar ratio of SPDP to IgG added to conjugation reaction mixtures is indicated

[b] Conjugation reaction products were separated by size by HPLC gel filtration on TSK 3000 SW. Integrated peak areas are shown here for the species of IgG eluting from the column

lar-mass conjugates. The ratio of the dimeric heteroconjugate to trimer and oligomer was substantially greater in this experiment, but the overall yield of conjugate was only 26%, and thus the yield of dimeric heteroconjugate was 15%. In a similar experiment BABR1 was derivatized with an equimolar ratio of SPDP and conjugated with OKT3, which had been derivatized with a twofold molar excess of SPDP. Here the extent of conjugation (31%) was lower than for the conjugation using twofold SPDP for both antibodies, but the yield of dimer was essentially the same. The 31% yield of conjugate is significantly greater than that in which equimolar SPDP was used for OKT3 (26%), suggesting that the pyridine disulfide group on BABR1 is more accessible for thiol-disulfide exchange than the pyridine disulfide on OKT3.

Isoelectric focusing

The isoelectric points of native and SPDP-modified OKT3 and BABR1 were compared with those of T3T3 and BR1BR1 homoconjugates and T3BR1 (Fig. 5). The pI of SPDP-OKT3 was 6.2–6.7. These bands were about 0.5 pI unit below those of native OKT3 and were more heterogeneous than OKT3. Band heterogeneity is most likely due to the modification of 0, 1, 2, 3, or 4 lysines (average = 2) and the variations in pK_a of the lysines modified. The pI of SPDP-BABR1 was 4.9–5.4, a decrease of about 0.3 pI unit in charge compared with BABR1. An increase in band heterogeneity was also observed. T3BR1 showed bands in the region of pI 5.2–5.7, intermediate in charge between those of SPDP-OKT3 and SPDP-BABR1, as expected of a true heteroconjugate. The absence of many bands of SPDP-OKT3 or SPDP-BABR1 in the heteroconjugate indicates that no homoconjugate is detectable, since T3T3 homoconjugates and BR1BR1 homoconjugates have pI values identical to those of SPDP-OKT3 and SPDP-BABR1, respectively. A silver-stained gel of these proteins (Fig. 5) confirmed that T3BR1 is free of homoconjugates. When T3BR1 was reduced and alkylated bands corresponding to SPDP-BABR1 and SPDP-OKT3 were observed in approximately equal proportion, as expected of a heteroconjugate [4].

Radial immunodiffusion

Single radial immunodiffusion analyses of two different T3BR1 preparations were performed as described above.

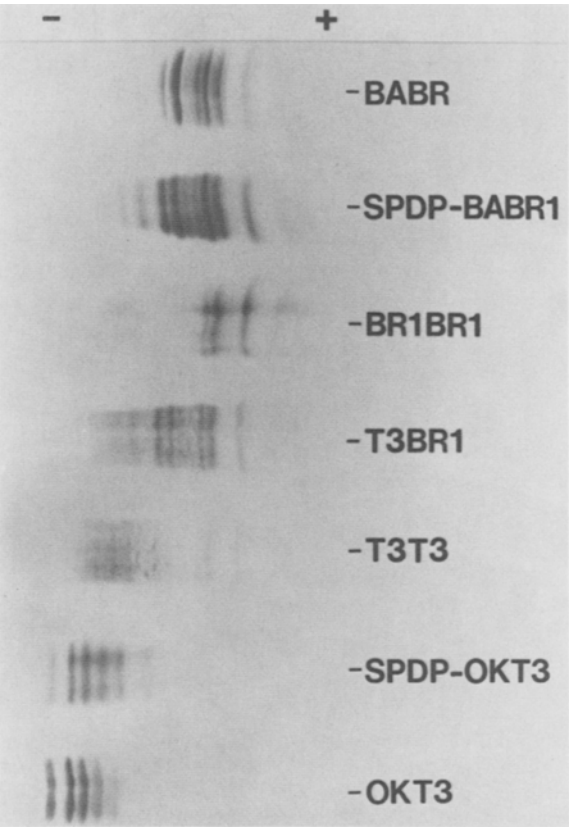

Fig. 5. Isoelectric focusing of derivatized monoclonal antibodies, native antibodies, homoconjugates and heteroconjugate. SPDP-OKT 3, T3T3 homoconjugate T3BR1 heteroconjugate, BR1BR1 homoconjugate, SPDP-BABR1 and BABR1 (20 μg each) were applied to an isoelectric focusing gel (5% acrylamide, 0.8% bisacrylamide, 13.3% glycerol, 1% Triton X-100, 0.5% riboflavin 5'-phosphate, and 5% ampholine pH 3.5–10), focused, and silver-stained as described in Materials and methods

The results confirmed the presence of both antibodies in approximately equal proportion: 129 μg/ml IgG1 and 162 μg/ml IgG2a; 117 μg/ml IgG1 and 100 μg/ml IgG2a.

Flow cytometry

We compared the functional binding and specificity of T3BR1 to OKT3 and BABR1 by indirect flow cytometry. The results are shown in Table 2. OKT3 bound specifically to normal (81%) and rIL-2 activated (84%) lymphocytes, with little nonspecific binding to tumour cells. BABR1 bound to 97%–100% of the breast carcinoma cells tested (BT-20, BT-474, ZR-75-1, and MCF-7) and also to 100% of WiDr, a colon carcinoma cell line. Low levels of nonspecific T3BR1 binding (8%–10%) were observed for the two melanoma cell lines (SK-MEL-1 and CaCL 78-4). T3BR1 bound essentially the same percentage of T cells and tumour cells as was bound by each parent antibody, thus confirming the bifunctionality of this heteroconjugate preparation. T3BR1 recognized about 50% less antigen than OKT3 on rIL-2-activated lymphocytes. T3BR1 bound at least 80% as much tumour cell-surface antigen as BABR1. These flow cytometry studies confirm that T3BR1 is a bifunctional heteroconjugate with binding specificities similar to those of its parent antibodies.

Table 2. Binding specificity of OKT3, T3BR1, and BABR1 to lymphocytes and tumor cell lines[a]

Cell line	Antibody binding (%)			
	OKT3	T3BR1	BABR1	MuIg
Normal PBL	81.2 (91)	82.7 (86)	2.0 (−)	1.6 (−)
rIL-2-activated lymphocytes	84.3 (174)	84.0 (135)	1.6 (−)	0.9 (−)
BT-20	2.3 (−)	98.5 (78)	99.5 (100)	1.6 (−)
BT-474	1.8 (−)	99.6 (140)	99.8 (141)	3.5 (−)
ZR-75-1	0.6 (−)	95.7 (87)	98.5 (94)	1.4 (−)
MCF-7	0.6 (−)	98.4 (118)	97.0 (98)	1.3 (−)
WiDr	1.1 (−)	99.9 (131)	99.8 (137)	1.1 (−)
SK-Mel-1	2.5 (−)	9.8 (24)	9.3 (18)	2.0 (−)
CaCL 78-4	8.0 (17)	8.9 (23)	7.6 (27)	1.9 (−)

[a] Antibody binding was measured by flow cytometry to normal and recombinant-interleukin-2(rIL-2)-activated PBL, breast carcinoma cell lines (BT-20, BT-474, ZR-75-1, and MCF-7), a colon carcinoma cell line (WiDr), and melanoma cell lines (SK-Mel-1 and CaCL 78-4). The binding of murine Ig (MuIg) was measured as a negative control. The results are expressed as the percentage positive, under which the mean fluorescence intensity is given in parentheses

T3BR1 heteroconjugates prepared using OKT3 and BABR1 F(ab')$_2$ fragments

F(ab')$_2$ fragments were prepared using purified OKT3 and BABR1 by pepsin digestion as described above. F(ab')$_2$ fragments were purified by HPLC gel filtration on TSK 3000 SW and protein A affinity chromatography, and conjugated using a threefold molar excess of SPDP as described above for intact IgG. The reaction mixture was chromatographed on TSK 3000 SW as shown in Fig. 6. Monomeric F(ab')$_2$ eluted at 56 min. The principal conjugation products were low-molecular-mass F(ab')$_2$ conjugates eluting in a broad peak at 43 min retention time. This material was determined to be F(ab')$_2$ conjugates by nonreducing electrophoresis (Fig. 7). No clearly discernable bands were observed for dimeric or trimeric F(ab')$_2$ heteroconjugates. Reducing electrophoresis of these fractions yielded bands of 25–30 kDa as expected for reduced F(ab')$_2$ (data not shown). The F(ab')$_2$ conjugates were determined to contain functional binding sites of both antibodies by flow cytometric analysis, and they stimulated lysis of BT-20 target cells in standard ^{51}Cr-release assays (data not shown). Therefore, the F(ab')$_2$ preparations as well as intact IgG yielded true heteroconjugates upon SPDP cross-linking.

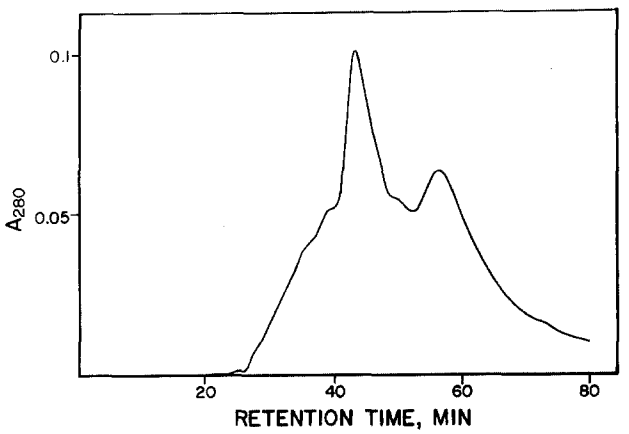

Fig. 6. HPLC gel filtration of T3BR1 F(ab')$_2$ heteroconjugate preparation. F(ab')$_2$ of both OKT3 and BABR1 were derivatized with a threefold molar excess of SPDP as described in the text. The conjugation reaction mixture was chromatographed on a TSK 3000 SW column. The peak at 56 min retention time is F(ab')$_2$ monomer

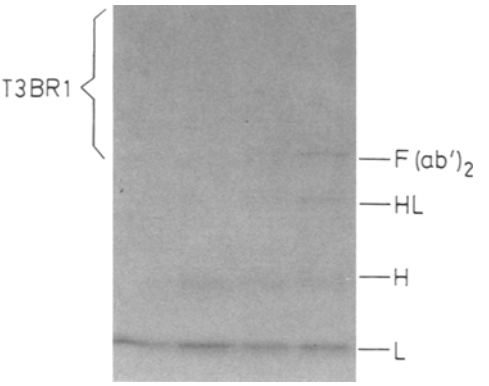

Fig. 7. Nonreducing electrophoresis of T3BR1 F(ab')$_2$ heteroconjugates. HPLC fractions shown in Fig. 6 were pooled and analyzed by nonreducing electrophoresis as described in the text. The gel migrations of F(ab')$_2$, half molecules (*HL*), heavy chains (*H*) and light chains (*L*) are indicated

Discussion

The simplest approach for the preparation of bifunctional antibodies is to cross-link the two purified antibodies chemically using a heterobifunctional agent such as SPDP. The chemistry of SPDP conjugation strongly favors formation of heteroconjugates over homoconjugates [4], and the ratio of SPDP to protein can be varied to control the extent of conjugation and the size of the product.

In this report we describe the conjugation of monoclonal antibodies BABR1 [anti-(breast carcinoma) IgG1] and OKT3 (anti-CD3 IgG2a) using modifications of the SPDP conjugation method designed to improve the purity and yield of T3BR1 dimers. This procedure employs very mild reducing conditions in order to avoid reduction of IgG disulfide bonds. We examined the reduction of SPDP-BABR1 spectrophotometrically in a dithiothreitol titration experiment (Fig. 1). It demonstrated complete reduction of pyridine disulfide groups at a dithiothreitol concentration of 160 μM, which is much lower than the dithiothreitol concentrations (15–25 mM) generally used by other investigators [4, 11, 14]. Relatively high concentrations of dithiothreitol might reduce native disulfide bonds in IgG molecules, and the extent of such reduction varies consider-

ably among different monoclonal antibodies. In our experience the native disulfide bonds of OKT3 heavy chains are very sensitive to reduction by dithiothreitol at concentrations as low as 1 mM (data not shown). Thus we have routinely reduced SPDP-BABR1 instead of SPDP-OKT3, and we routinely perform reductions at dithiothreitol concentration of 450–900 μM to obtain complete reduction of pyridine disulfide groups without reducing IgG native disulfide bonds.

The conjugation reaction of SPDP-OKT3 and reduced SPDP-BABR1 was performed at 37° C and monitored spectrophotometrically (Fig. 2). The reaction was essentially complete after 50 min incubation. Therefore, we routinely incubate conjugation reaction mixtures for 1–1.5 h, monitoring the reaction spectrophotometrically until completion. Thus the duration of incubation used here is substantially less than that of reactions performed at room temperature, which may be as long as 4 h [11], thereby reducing the possibility of disulfide formation by oxidation of sulfhydryls.

We used HPLC gel filtration on a TSK 3000 SW column to separate heteroconjugates and unconjugated IgG. The preparative TSK 3000 SW columns (21.5 × 600 mm) used in this study contain 26000–34000 theoretical plates and, therefore, provide excellent resolution of proteins by size (Fig. 3). In our experience HPLC gel filtration enables purification of heteroconjugates more rapidly and with greater resolution and less protein dilution than gel filtration by conventional liquid chromatography media, such as AcA22 or Sephacryl S-300, which have been used to date in other studies of IgG heteroconjugates [10, 11, 22]. IgG monomers and dimers were almost completely separated and dimers and trimers were mostly resolved (Fig. 3). The identities of these molecular species were elucidated from comparisons of their migration on nonreducing polyacrylamide gels (Fig. 4) and their elution from TSK 3000 SW. IgG dimers and trimers are not resolved by conventional gel filtration procedures [11, 17]. TSK 3000 SW routinely yields preparations of dimeric heteroconjugates more than 80% pure, compared to about 60% purity with AcA22 [17].

We tested several different ratios of SPDP to IgG in order to optimize the yield of T3BR1 dimers (Table 1). The use of twofold molar excess SPDP resulted in a much better yield of dimer than the use of a threefold molar ratio of SPDP. A comparable yield of dimers was obtained with the use of twofold molar SPDP for OKT3 and equimolar SPDP for BABR1. However, the protocol using threefold molar SPDP for BABR1 and equimolar SPDP for OKT3 produced a significantly lower yield of dimers. Using a twofold molar ratio of SPDP to IgG we routinely obtained yields of 30% dimeric heteroconjugate, a significantly greater yield than the 10%–20% yields obtained by other investigators using higher molar ratios of cross-linker to IgG [11, 22].

The dimeric T3BR1 preparations have been analyzed by isoelectric focusing (Fig. 5). The results indicate that the native T3BR1 has a pI (5.2–5.7) intermediate between those of OKT3 (6.2–6.7) and BABR1 (4.9–5.4), as predicted for a true heteroconjugate. No homoconjugates with the same pI as parent antibodies were observed in these preparations. Reduced and alkylated samples of T3BR1 contained bands of the same pI as were observed with the parent antibodies (data not shown).

Flow cytometry results (Table 2) confirmed that functional binding sites of both antibodies were present in T3BR1 heteroconjugates. T3BR1 and BABR1 bind equally well to several carcinoma cell lines of breast and colon origin, consistent with the published reactivity of BABR1 to carcinoma cells derived from different organs. T3BR1 also bound T cells to an extent comparable to OKT3 binding. Therefore, the T3BR1 heteroconjugate is bifunctional. Furthermore, the specificity of T3BR1 for binding to cell-surface antigens was the same as the specificities of OKT3 and BABR1. Also, we have recently shown by dual fluorescence flow cytometry that T3BR1 is a bifunctional heteroconjugate (Price, J. O. and West, W. H., unpublished data). In these experiments anti-IgG1 and anti-IgG2a labeled with fluorescein isothiocyanate and phycoerythrin, respectively, were observed to bind to both lymphokine-activated killer cells and BT-20 breast carcinoma cells only in the presence of T3BR1 heteroconjugate.

A number of reports have described the preparation of bifunctional antibodies by chemically cross-linking proteolytic fragments of the two parent antibodies [3, 9, 11, 18, 20, 25]. We also have prepared bifunctional heteroconjugates by cross-linking $F(ab')_2$ of both OKT3 and BABR1 with SPDP (Figs. 6, 7). The major disadvantages to this approach are low yield and significant loss of binding activity after proteolysis.

We have also shown that T3BR1 heteroconjugate preparations enhance lysis of BT-20 targets in 4-h ^{51}Cr-release assays using rIL-2-activated lymphocytes as effectors [15]. Heteroconjugates, prepared using $F(ab')_2$ fragments of both BABR1 and OKT3, also enhanced lysis of BT-20 targets to a similar extent, thus confirming that the F_c region of the two antibodies is not required for enhancement of target cell lysis. These results suggest that heteroconjugates of OKT3 and antitumour antibodies may be a therapeutically beneficial approach for targeting lymphokine-activated killer cells.

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References

1. Barr IG, McDonald HR, Buchegger F, von Fliedner V (1987) Lysis of tumour cells by the retargeting of murine cytolytic T lymphocytes with bispecific antibodies. Int J Cancer 40: 423
2. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem 72: 248
3. Brennan M, Davidson PF, Paulus H (1985) Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G1 fragments. Science 229: 81
4. Bush D, Winkler MA (1989) Iso electric focusing of cross-linked monoclonal antibody heterodimers, homodimers, and derivatized antibodies. J Chromatog (Biomed Appl) 489: 303
5. Carlsson J, Drevin H, Axen R (1978) Protein thiolation and reversible protein-protein conjugation. Biochem J 173: 723
6. Clausen J (1969) In: Work TS, Work E (eds) Laboratory techniques in biochemistry and molecular biology. North-Holland, Amsterdam, p 423

7. Dulbecco R, Vogt M (1954) Plaque formation and isolation of pure lines with poliomyelitis viruses. J Exp Med 99: 167
8. Ey PL, Prowse SJ, Jenkin CR (1978) Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. Immunochemistry 15: 429
9. Glennie MJ, McBride HM, Worth AT, Stevenson GT (1987) Preparation and performance of bispecific $F(ab'r)_2$ antibody containing thioether-linked Fab'r fragments. J Immunol 139: 2367
10. Jung G, Honsik CJ, Reisfeld RA, Muller-Eberhard HJ (1986) Activation of human peripheral blood mononuclear cells by anti-T2: killing of tumor target cells coated with anti-target anti-T3 conjugates. Proc Natl Acad Sci USA 83: 4479
11. Karpovsky B, Titus JA, Stephany DA, Segal DM (1984) Production of target-specific effector cells using hetero-cross-linked aggregates containing anti-target cell and anti-Fcr receptor antibodies. J Exp Med 160: 1686
12. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriaphage T4. Nature 227: 680
13. Liu MA, Kranz DM, Kurnick JT, Boyle LA, Levy R, Eisen HN (1985) Heteroantibody duplexes target cells for lysis by cytotoxic T lymphocytes. Proc Natl Acad Sci USA 82: 8648
14. Lotze MT, Roberts K, Custer MC, Segal DA, Rosenberg SA (1987) Specific binding and lysis of human melanoma by IL-2-activated cells coated with anti-T3 of anti-Fc receptor cross-linked to antimelanoma antibody: a possible approach to the immunotherapy of human tumours. J Surg Res 42: 580
15. Marshall GD, Foglesong PD, Price JO, Winkler MA, West WH (1988) Increased antigen-specific activity of interleukin-2 activated cells by heteroconjugate antibodies. Immunology and Allergy Clinics of North America 8: 126
16. Morrissey JH (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem 117: 307
17. Perez P, Hoffman RW, Shaw S, Bluestone JA, Segal DM (1985) Specific targeting of cytotoxic T cells by anti-T3 linked to anti-target cell antibody. Nature 316: 354
18. Perez P, Hoffman RW, Titus JA, Segal DM (1986) Specific targeting of human peripheral blood T cells by heteroconjugates containing anti-T3 cross-linked to anti-target cell antibodies. J Exp Med 163: 166
19. Perez P, Titus JA, Lotze MT, Cuttita F, Longo DL, Groves EL, Rabin H, Durda PJ, Segal DM (1986) Specific lysis of human tumour cells by T cells coated with anti-T3 cross-linked to anti-tumour antibody. J Immunol 137: 2069
20. Raso V, Griffin T (1981) Hybrid antibodies with dual specificity for the delivery of ricin to immunoglobulin-bearing target cells. Cancer Res 41: 2073
21. Rosenberg SA, Lotze MT, Muul LM, Laitman S, Chang AE, Ettinghausen SE, Matory YL, Skibber JM, Shilani E, Vetto JT, Seipp CA, Simpson C, Reichert CM (1985) Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N Engl J Med 313: 1485
22. Scott CF, Lambert JM, Kalish RS, Morimoto C, Schlossman SF (1988) Human T cell can be directed to lyse tumour targets through the alternative activation/T11-E rosette receptor pathway. J Immunol 140: 8
23. Staerz UD, Kanagawa O, Bevan MJ (1985) Hybrid antibodies can target sites for attack by T cells. Nature 314: 628
24. Steinbuck M, Andran R (1969) The isolation of IgG from mammalian sera with the aid of caprylic acid. Arch Biochem Biophys 134: 279
25. Stevenson GT, Glennie MJ, Paul FE, Stevenson FK, Watts HF, Wyeth P (1985) Preparation and properties of FabIgG, a chimeric univalent antibody designed to attack tumour cells. Biosci Rep 5: 991
26. Stuchbury T, Shipton M, Norris R, Malthouse JPG, Brocklehurst K, Herbert JAL, Suschitzky H (1975) A reporter group delivery system with both absolute and selective specificity for thiol groups and an improved fluorescent probe containing the 7-nitrobenzo-2-oxa-1,3-diazole moiety. Biochem J 151: 417
27. Titus JA, Garrido MA, Hecht TT, Winkler DF, Wunderlich JR, Segal DM (1987) Human T cells targeted with anti-T3 cross-linked to antitumour antibody prevent tumour growth in nuce mice. J Immunol 138: 4018
28. Titus JA, Perez P, Kaubisch A, Garrido MA and Segal DM (1987) Human K/natural killer cells targeted with hetero-cross-linked antibodies specifically lyse tumour cells in vitro and prevent tumour growth in vivo. J Immunol 139: 3153
29. West WH, Tauer KW, Yannelli JR, Marshall GD, Orr DW, Thurman GB, and Oldham RK (1987) Constant-infusion recombinant interleukin-2 in adoptive immunotherapy of advanced cancer. N Engl J Med 316: 898

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