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ISOELECTRIC FOCUSING OF CROSS-LINKED MONOCLONAL ANTIBODY HETERODIMERS, HOMODIMERS AND DERIVATIZED MONOCLONAL ANTIBODIES

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SUMMARY

Anti-tumor monoclonal antibodies were cross-linked to the anti-CD3 T-cell antibody OKT3 by the use of the heterobifunctional cross-linker succinimidyl-3-(2-pyridyldithio)proprionate. Derivatized monoclonal antibodies, heterodimers, and homodimers were resolved by analytical isoelectric focusing in polyacrylamide gels containing 1% Triton X-100. Isoelectric points of the derivatized antibodies were lower than native antibodies, consistent with lysine derivatization. Antibodies derivatized with 2-iminothiolane were equivalent or slightly higher in p*I* compared with native antibodies. Heterodimers focused in microheterogeneous bands between the p*I* extremes of the parent derivatized antibodies. The isoelectric points of homodimers were lower than those of parental antibodies and could be distinguished from heterodimers. Reduced and alkylated heterodimers were resolved into their constituent antibodies by isoelectric focusing.

INTRODUCTION

The uses of monoclonal antibodies as therapeutic or diagnostic reagents can be increased by chemically cross-linking two different monoclonal antibodies to create a heterodimer antibody with dual specificity. Anti-tumor antibodies conjugated to anti-CD3 T cell antibodies have been shown in vitro [1–3], and in vivo [4] to target cytotoxic cells to tumor cells, enhancing tumor cell lysis. Antibodies to the Fc receptor coupled to antitumor antibodies were similarly effective [5]. An anti-breast tumor antibody cross-linked to OKT3 enhances the killing in vitro of breast tumor cells by interleukin-2 (IL-2)-activated leukocytes from patients undergoing IL-2 therapy [6]. We have chosen to use chemical cross-linking to initially make heteroconjugates instead of hybrid–hybridoma procedures [7] for two reasons. First, chemically cross-linked heteroconjugates can be synthesized

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and purified in a few days, while hybrid–hybridomas require months to produce drug-resistant cell lines and to fuse and clone the appropriate secretor cells. Second, chemically produced heteroconjugate dimers are larger than parental antibodies and can be readily purified by size-exclusion; molecules made by hybrid–hybridomas are the same size as parental molecules and purification is difficult if parental molecules are similar in charge and/or hydrophobicity. For such chemical heteroconjugates to be therapeutically active, however, they must be free from significant amounts of homoconjugated antibodies or from derivatized uncojugated antibodies, which compete against heteroconjugate for binding sites. Isoelectric focusing (IEF) has been used to distinguish parental antibodies from the hybrid antibodies produced by heterohybridomas [8,9]. This paper describes procedures for IEF of derivatized antibodies, antibody heteroconjugates, and antibody homoconjugates. The methods described allow the detection of homodimers in heterodimer preparations. In addition, IEF of reduced and alkylated heterodimer can verify that heterodimers have been synthesized.

EXPERIMENTAL

Chemicals

The T-cell anti-CD3 monoclonal antibody OKT3 was supplied by Ortho Pharmaceutical (Raritan, NJ, U.S.A.); anti-breast tumor monoclonal antibody BR1 was produced by Biotherapeutics (Franklin, TN, U.S.A.) from clone B38.1 supplied by J. Schlom, NCI; N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), 2-iminothiolane hydrochloride, and Surfact-Amps X-100 (a specially purified grade of Triton X-100) were purchased from Pierce (Rockford, IL, U.S.A.).

Iodoacetamide was purchased from Eastman Kodak (Rochester, NY, U.S.A.). Dithiothreitol (DTT), ultrapure grade, was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Acrylamide, N,N′-methylenebisacrylamide, N,N,N′,N′-tetramethylenediamine (TEMED), IEF standards, and Coomassie R-250 were obtained from Bio-Rad (Richmond, CA, U.S.A.). Riboflavin-5′-phosphate, sodium salt, was purchased from Haake Buchler Instruments (Saddle Brook, NJ, U.S.A.). Sephadex G-25M (PD-10 columns, 9-ml bed volume) was purchased from Pharmacia (Piscataway, NJ, U.S.A.). Ampholine (pH 3.5–10) was obtained from LKB (Rockville, MD, U.S.A.). Radial immunodiffusion kits for mouse immunoglobulin G2a and G1 were purchased from ICN Immuno-Biologicals (Lisle, IL, U.S.A.).

Synthesis of heteroconjugates and homoconjugates

The antibody conjugates were prepared using one of two modifications of the method of Carlsson et al. [10]. In the first approach used for heteroconjugates, both parent antibodies were modified with SPDP to give pyridyl disulfide (PD) antibodies, and one of the PD antibodies was further reduced with DTT prior to reacting them together. In the second approach, used for OKT3 homoconjugates, one of the parent antibodies was reacted with 2-iminothiolane and the other was SPDP-modified before reacting them together. For SPDP modification, each parent antibody in phosphate-buffered saline (PBS) (0.002 *M* disodium hydrogen-

phosphate, 0.008 *M* sodium dihydrogenphosphate, and 0.15 *M* sodium chloride, pH 7.4) was reacted with two-fold molar excess SPDP at room temperature for 30 min and desalted on a PD-10 column equilibrated in PBS–EDTA (PBS with 1 m*M* EDTA). The moles of SPDP per mole of IgG were determined spectrophotometrically by monitoring the release of pyridylthione at 343 nm in an aliquot to which DTT was added to a final concentration of 25 m*M*. The molar extinction coefficient (ϵ) for pyridylthione is 8080 $mol^{-1}\ cm^{-1}$ ($E^{1\%}_{1cm,\ 343\ nm} = 727$). One of the PD antibodies was dialyzed for 18 h at 4°C against 0.1 *M* sodium acetate buffer with 1 m*M* EDTA (pH 4.2). The dialyzed antibody was then reduced with 1.5 m*M* DTT. The extent of reduction was checked by measuring pyridylthione release as described above. When the reduction was complete as evidenced by no further absorbance increase, the antibody was desalted on a PD-10 column equilibrated with PBS–EDTA. The two parent antibodies were then mixed together and incubated at 37°C for 1 h. Dimer heteroconjugate or homoconjugate was separated from high-molecular mass conjugates and unreacted monomers on a TSK 3000 SW 60 cm×2.5 cm I.D. column using a Beckman high-performance liquid chromatograph, 60–80 mg of protein were loaded onto the column. The elution buffer was 0.1 *M* sodium acetate containing 1 m*M* EDTA, pH 6.8. The flow rate was 2.0 ml/min, and 2-ml fractions were collected.

Iminothiolated parent antibody was prepared by reacting the antibody (in 50 m*M* Tris–HCl, 0.15 *M* sodium chloride, and 1 m*M* EDTA, pH 8.0) with a two- to five-fold excess of 2-iminothiolane under nitrogen for 45 min at room temperature. Iminothiolated antibody was desalted on a PD-10 column equilibrated with PBS–EDTA. The extent of iminothiolation was checked using Ellman's reaction [11]. The iminothiolated parent antibody was reacted with an SPDP-modified parent antibody at 37°C for 1 h and separated by HPLC as described above. Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) was used to confirm that the heteroconjugate was not significantly contaminated by high-relative-molecular-mass conjugates or unreacted monomers (data not shown). When the parent antibodies were of different IgG subclasses, radial immunodiffusion, performed according to the kits' instructions, was used to quantitate the relative amounts of each parent present in the heteroconjugate.

Reduction and alkylation of antibodies

Antibody or SPDP-derivatized antibody (250 μg) was diluted 1:10 with 1 *M* Tris and 0.1 *M* DTT (pH 8.0) and incubated for 30 min at room temperature. Iodoacetamide was then added to a final concentration of 0.25 *M* and the reaction allowed to proceed for 30 min at room temperature in the dark. The reduced and alkylated antibody was desalted on a PD-10 column equilibrated with PBS diluted 1:2 with water and concentrated by centrifugation at 2000 *g* in a Centricon-10 device (Amicon) to at least 1 mg/ml protein prior to application to the gel.

Isoelectric focusing

Focusing was done in a polyacrylamide gel 0.7 mm thick on a Pharmacia FBE-3000 with a Hoefer PS 1200 DC power supply. The gel contained 6.0% acrylamide,

0.16%, N,N′-methylenebisacrylamide, 13.7% glycerol, 1% Triton X-100, 5% Ampholine (pH 3.5–10), 10 n*M* riboflavin-5′-phosphate, and TEMED as an accelerator. Some of the gels also contained 6 *M* urea. After polymerization, the gel was prefocused at 30-W constant power for 1 h at 10°C using 50 m*M* sodium hydroxide as the catholyte and 50 m*M* acetic acid as the anolyte. A 10–15 μg sample was applied to gels to be silver-stained, and 15–30 μg were applied to gels to be Coomassie-stained. IEF standards, 5–10 μl of a 1:5 dilution with water, were run on all gels. The gels were run at a constant voltage of 1400 V for 2.5 h, with the sample pads being removed after 45 min. The surface pH was measured with an Ingold combination pH surface electrode, 3-mm tip on a Beckman pH meter. The gels were then fixed in 10% trichloroacetic acid, 4% sulfosalicylic acid for 1 h and subjected to three 20-min washes with deionized water. Gels were stored overnight in 200–300 ml of deionized water. Gels were silver-stained according to the method of Morrissey [12] or with 0.04% Coomassie Blue R-250, 27% 2-propanol, 10% acetic acid, and 0.5% copper sulfate for 1 h and destained with 12% 2-propanol, 7% acetic acid, and 0.5% copper sulfate. The pH values of the bands in each lot of IEF standards were calculated from curves constructed from surface pH electrode measurements, and checked against values supplied by the manufacturer. The positions of bands and regions of microheterogeneity of each sample were measured, and the associated pH values calculated from the surface pH measurement curves. In gels where surface pH measurements were not done, the pH values were calculated from curves constructed from the IEF standards. No pH measurements were taken from gels containing urea.

RESULTS

To reproducibly resolve derivatized or cross-linked antibodies, we found it necessary to make two modifications in focusing procedures for immunoglobulins [13].

(1) Triton X-100, the non-ionic detergent used to prevent precipitation of immunoglobulins at their isoelectric points, must be of a high purity grade, low in

TABLE I

ISOELECTRIC POINTS OF DERIVATIZED OR CROSS-LINKED MONOCLONAL ANTIBODIES

At least three determinations were made and the means are shown. Standard deviations were less than 0.5, with the exception of OKT 3×BR1, which was 0.8.

Antibody or conjugate	p*I* range
OKT3	6.9–7.6
OKT3-PD	6.7–7.5
OKT3-SH	7.2–7.8
OKT3×OKT3	6.5–7.3
OKT3×BR1	6.1–6 8
BR1	6.3–6.8
BR1-PD	5 6–6.7
BR1×BR1	5.4–6.6

peroxides and carbonyl compounds. SPDP-derivatized antibodies or cross-linked heteroconjugates focused in gels containing less purified grades of Triton X-100 showed artifactual bands close to the anode (data not shown). These bands may be due to reaction of derivatized antibody with Triton X-100 contaminants such as peroxides or carbonyls.

(2) Derivatized antibodies (but not heteroconjugates) were reduced and alkylated before IEF to prevent further reaction or disulfide interchange on storage. Reduction and alkylation had no significant effect on isoelectric points compared with unreduced and unalkylated antibodies.

Monoclonal anti-breast tumor antibody BR1 showed nine bands on IEF, and OKT3 showed five bands. The higher number of bands in BR1 is probably due to expression of two different light chains by this clone (unpublished results). The p*I* values of the lowest and highest bands were determined (Table I). SPDP derivatization of OKT3 resulted in OKT3-PD with an average of 1.5 PD per IgG. OKT3-PD showed bands which were shifted 0.1–0.2 p*I* unit towards the anode (Fig. 1), as expected of the modification of one to four lysines, neutralizing the

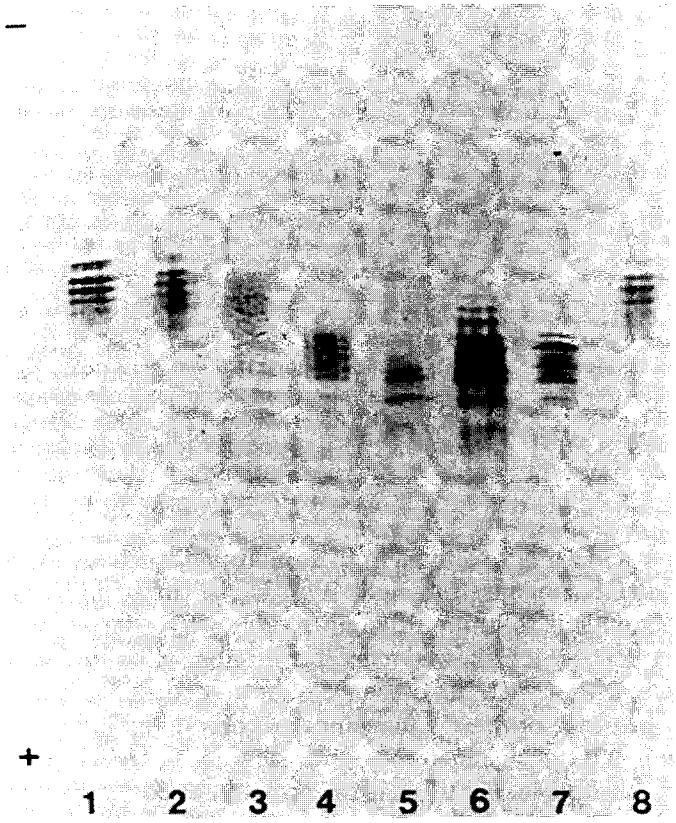

Fig. 1. IEF of derivatized antibodies, heterodimers and homodimers. IEF was in polyacrylamide gels containing ampholytes 3.5–10, 1% Triton X-100, and 6 *M* urea as described in Experimental, 20 μg of each sample were focused. Lanes: 1 = OKT3; 2 = OKT3-PD; 3 = OKT3 × OKT 3; 4 = OKT3 × BR1; 5 = BR1 × BR1; 6 = BR1-PD; 7 = BR1; 8 = OKT3.

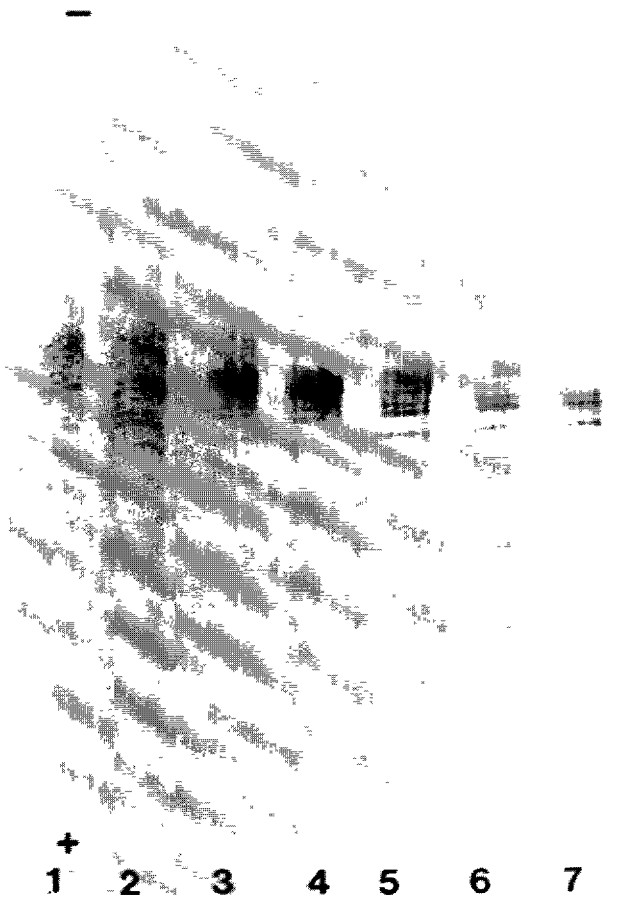

Fig 2 IEF of heterodimers mixed with varying amounts of homodimers. IEF was done on a polyacrylamide gel with 6 *M* urea as described in Experimental, keeping the total amount of antibody mixture at 20 μg. Lanes: 1 = 100% OKT3×OKT3; 2 = 70% OKT3×OKT3 and 30% OKT3×BR1, 3 = 40% OKT3×OKT 3 and 60% OKT3×BR1; 4 = 100% OKT3×BR1; 5 = 40% Br1×Br1 and 60% OKT3×BR1; 6 = 70% BR1×BR1 and 30% OKT3×BR1; 7 = 100% BR1×BR1.

positive charge on this amino acid. Bands equivalent to unmodified antibody were also seen. Iminothiolation of OKT3 caused a slight increase in the p*I* of some bands, but was otherwise equivalent to unmodified antibody. SPDP derivatization of BR1 resulted in BR1–PD, which showed a greater number of bands than non-derivatized antibody, consistent with the presence of both modified and unmodified chains. The wider p*I* range of these bands allowed their detection compared with OKT3–PD, which had tightly clustered bands. Similar to OKT3–PD, derivatized BR1 bands were shifted ca. 0.2 p*I* unit towards the anode. A few of the BR1–PD bands were shifted to the cathode in some preparations, but this result was not consistent.

Homoconjugates or heteroconjugates showed microheterogeneity on IEF to the extent that individual bands were difficult to distinguish (Fig. 1). The p*I* values

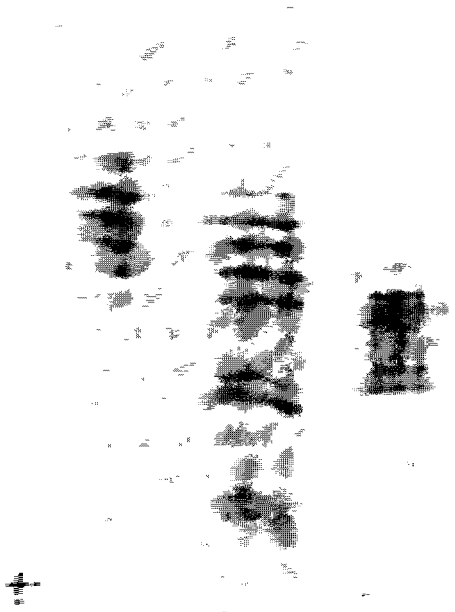

Fig. 3. IEF of reduced and alkylated heteroconjugate compared with reduced and alkylated PD antibodies. Lanes: left, OKT3–PD; middle, OKT3×BR1, right, BR1–PD. The anode is at the bottom.

of the lower and upper limits of this microheterogeneity region were measured for each pattern (Table I). Heteroconjugates had lower limits between the lower bands of parent PD antibodies. The upper limit of heteroconjugate was also between the upper limits of parental PD antibodies. For example, OKT3×BR1 had a lower limit of 6.1, between 6.7 of OKT3–PD and 5.6 of BR1–PD. The lower and upper p*I* limits of homoconjugate OKT3×OKT3 were 0.2 p*I* units below those of the parental OKT3–PD. BR1×BR1 homodimer had lower and upper limits 0.1–0.2 p*I* units below those of BR1–PD. These differences made it possible to detect OKT3 homodimer in the OKT3×BR1 heterodimer preparations. To demonstrate this, varying amounts of homodimer BR1×BR1 or OKT3×OKT3 were added to BR1×OKT3 heterodimer such that a final ratio of 7:3 or 4:6 for homodimer/heterodimer were obtained. Fig. 2 shows that at each of these levels, OKT3×OKT3 homodimer shows prominently as bands above the heterodimer. The lowest bands of BR1×BR1 homodimer focused below the heterodimer bands and showed in varying amounts when added to heterodimer. Because most of the BR1 homodimer focused in the region of the heterodimer, we checked heteroconjugates by another method, radial immunodiffusion, which permitted to quantitate the amounts of IgG1 (BR1) and IgG2a (OKT3). The molar ratio of the two antibodies, IgG1/IgG2a, was 47:53 in heteroconjugate. Thus the two antibodies were approximately equimolar, and since OKT3 homodimer was not detected, we inferred that BR1 homodimer could not be present in significant amounts. If there had been contaminating BR1×BR1 homodimer and no OKT3×OKT3, the ratio of the two antibodies would not have been close to 1:1.

Reduction and alkylation of heterodimer OKT3×BR1 separated the immunoglobulins into bands typical of the two parental PD antibodies (Fig. 3). Equiv-

alent amounts of BR1 and OKT3 bands are present upon reduction and alkylation, as expected from the molar ratio found by radial immunodiffusion.

DISCUSSION

The unmodified monoclonal antibodies used in this study showed five to nine bands on silver-stained gels. The heterogeneity in the number of bands of monoclonal antibodies has been ascribed to amidation/deamidation of glutamine or asparagine or to variability in carbohydrate content [14,15]. Modification with SPDP to give PD antibody caused a decrease in the p*I* values of some of the bands and increased the heterogeneity of most of the bands, as expected from the neutralization of charge(s) of one to four lysines per molecule. The bands closest to the cathode showed no decrease in p*I* on modification, and they were less intensely stained, implying that some of the antibody molecules were not modified. As shown by Gennis and Cantor [16], the percentages of unmodified and modified antibodies should be approximated by a Poisson distribution, since 30–60 lysines are available to react and only a few sites per antibody are modified. For antibody with an average of two PD groups per molecule, calculation from the Poisson distribution predicts ca. 22% of the antibody would be unmodified and less than 5% would have more than four lysines per molecule. Several bands of iminothiolated OKT3 showed a slight but reproducible increase in p*I*, which was unexpected. Iminothiolation of lysines should not significantly change the charge, since it causes a substitution of a positively charged amidine moiety for an amine and conformational changes leading to an increase in charge are unlikely with this reagent [17]. One explanation may be modification of carbohydrates by 2-iminothiolane. 2-Iminothiolane has been shown to be reactive with polysaccharides [18]. Iminothiolation of antibody carbohydrate would result in a thiolated carbohydrate, and in some cases a positively charged amidine group could be retained.

Homodimer showed no bands equivalent to the highest p*I* bands of SPDP-modified antibody. This was expected since these bands of high p*I* are probably due to unmodified antibody (see above), and unmodified antibodies will not conjugate. Heterodimer showed much greater heterogeneity in the bands, and the p*I* range of the bands was between those of either parental SPDP-modified antibody. This result was expected, since heteroconjugate should show a charge which is an average of the charges of the parental antibodies. The microheterogeneity of heterodimer is most likely due to many of the ten different possible pairings of antibodies with one to four modified lysines. The difference between the IEF patterns of the heterodimer and the homodimers enabled us to detect homodimer in preparations of heterodimer. In other cases where homodimer overlapped the heterodimer microheterogeneity region, radial immunodiffusion data enabled us to exclude the possibility of significant homoconjugate content.

IEF of reduced and alkylated heterodimer showed the IEF patterns of the two parental SPDP-modified antibodies. Mild reduction and alkylation of the parental antibody caused no significant changes in banding patterns.

Protein heteroconjugates have potential for a variety of diagnostic or thera-

peutic uses. The IEF procedures described here may be generally applicable to analysis of protein heteroconjugates which cannot be distinguished from homoconjugates by size and which have components of similar molecular mass and differing isoelectric points.

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