RNase P RNA did not serve as an RNA ligase substrate. The oxidized RNA bonds contained less than 2 percent of the [^{32}P]pCp incorporated into the unmodified RNA, as determined by scintillation counting of gel slices and remained intact, as judged by silver staining (*4, 5*). Incubation of oxidized RNase P RNA in the high salt buffer of the RNase P assay had no effect on its later ability to serve as an RNA ligase substrate, demonstrating that the oxidized terminus is stable under the unusual ionic conditions of the assay. Furthermore, periodate-oxidized precursor tRNA could serve equally well as substrate for either native or oxidized RNase P RNA (*5*). Thus, the 3′ termini of neither the RNase P RNA nor its substrate are required for accurate processing. The mechanism of the RNase P reaction is different from those that occur during intron excision from *Tetrahymena* rRNA.

Since the 3′-OH of the RNase P RNA is not the initiating nucleophile in the RNase P processing reaction, it is likely that a hydroxyl group from water serves that role. The RNase P reaction had a significant dependence on hydroxide concentration (Fig. 1C) with an optimum at *p*H 8 to 8.5. The *p*H at the half-maximum reaction rate does not correlate with any of the reported *p*K values for nucleic acid ionizable groups, consistent with dependence on hydroxide concentration. Above an approximate *p*H of 9, all activity was lost, probably because of denaturation of the catalyst or substrate RNA's due to deprotonation of U-N3 (at *p*H 9 to 9.5).

Reactions involving hydroxide attack on phosphate esters are commonly catalyzed by proteins. The amino acid side chains may provide proton exchange mechanics to activate a water molecule positioned to attack the esterified phosphorus (Fig. 2). We envisage that the precursor tRNA substrate binds to the surface of the RNase P RNA in a manner analogous to the binding of a substrate to a protein surface, and that nucleic acid groups manipulate the reactants. A hydrated Mg^{+2}-coordinate complex offers attractive geometry for the reaction (*6*), but the actual catalysis must derive from the RNase P RNA. The proton donor-acceptor roles might be provided by nucleoside base tautomeric transitions, nonterminal 2-OH groups, or, conceivably, internucleotide phosphates with high *p*K values. The delineation of the active site, the identification of chemically important groups, and the roles of high concentrations of mono- and divalent cations required for the RNA reaction remain to be investigated.

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Preparation of Bispecific Antibodies by Chemical Recombination of Monoclonal Immunoglobulin G_1 Fragments

Abstract. *Preparation of bispecific antibodies by the chemical reassociation of monovalent fragments derived from monoclonal mouse immunoglobulin G_1 is inefficient because of side reactions during reoxidation of the multiple disulfide bonds linking the heavy chains. These side reactions can be avoided by using specific dithiol complexing agents such as arsenite and effecting disulfide formation with a thiol activating agent such as 5,5′-dithiobis(2-nitrobenzoic acid). In this way bispecific antibodies were obtained in high yield and free of monospecific contaminants from monoclonal mouse immunoglobulin G_1 fragments. The bispecific antibodies were used as agents for the selective immobilization of enzymes.*

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Bispecific antibodies, hybrid immunoglobulins with two different antigen-binding sites, have been prepared from polyclonal rabbit immunoglobulins (*1-3*). They have many potential uses, ranging from immunodiagnostic procedures to targeted delivery of drugs. Nevertheless, the applications of bispecific antibodies would be considerably enhanced if they could be derived from monoclonal antibodies. The preparation of bispecific monoclonal antibodies by fusion of antibody-producing cells was recently described by Milstein and Cuello (*4*). Although their procedure produces a mixture of hybrid antibodies with various assortments of chains, which must be fractionated to yield the desired bispecific molecules, they suggested that it is superior to a chemical reconstitution approach on account of certain technical problems (*4*). These problems include the need to dissociate immunoglobulins into half-molecules without damaging the antigen-binding sites and to reform three disulfide bonds linking the heavy chains, characteristic of mouse immunoglobulins, without allowing interfering side reactions, such as formation of disulfide bonds within chains. Nevertheless, it has been possible to obtain hybrid monoclonal antibodies in low yields (*5*). We report here a chemical procedure for preparing bispecific antibody fragments from monoclonal mouse immunoglobulin G_1 (IgG_1), a procedure that avoids these problems and generates the desired bispecific reagent in high yield as the only product, obviating the need for further purification.

To dissociate reduced immunoglobulin half-molecules under mild conditions, we used the method of Nisonoff and Mandy (*1*), who removed the Fc′ portion of rabbit IgG by limited pepsin hydrolysis to yield $F(ab')_2$. The cleavage of monoclonal mouse IgG_1 with pepsin to yield $F(ab')_2$ proceeded readily, provided that the reaction was carried out at a slightly lower *p*H than that with rabbit IgG (*6, 7*). About 80 percent of the monoclonal IgG_1's were converted to $F(ab')_2$ with a good yield (75 to 95 percent) by incubation with pepsin (2 percent by weight) in 0.1*M* sodium acetate (*p*H 4.2) for 18 hours at 37°C. On reduc-

tion of $F(ab')_2$ with 1 m*M* 2-mercaptoethylamine in 0.1*M* sodium phosphate (*p*H 6.8) and 1 m*M* EDTA for 18 hours at 25°C, complete conversion to Fab′ was observed.

Our attempts and those of others (*5*) to regenerate $F(ab')_2$ from Fab′ prepared as described above gave yields that were generally poor compared to those obtained with rabbit immunoglobulin fragments (*1–3*). This problem was probably related to the three disulfide bonds that bridge the heavy chains in mouse IgG (*8*) (in contrast to a single such bond in rabbit IgG), so that dimerization, which involves formation of disulfides between chains, must compete with disulfide formation within chains. Our strategy for avoiding this problem involved two major modifications of the procedure of Nisonoff and Mandy (*1*). The first consisted of carrying out the reduction of $F(ab')_2$ in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols (*9*) and impede intramolecular disulfide formation. The second involved activating the thiols of one of the Fab′ preparations as the thionitrobenzoate (TNB) derivative (*10*). The reaction scheme (Fig. 1) consists of the reduction of two different $F(ab)'_2$ fragments with 2-mercaptoethylamine in the presence of sodium arsenite and their conversion to the TNB derivatives by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent). One of the Fab′-TNB derivatives is then reconverted to the Fab′-thiol by reduction with 2-mercaptoethylamine and is mixed with an equimolar amount of the other Fab′-TNB derivative to form the hybrid dimer.

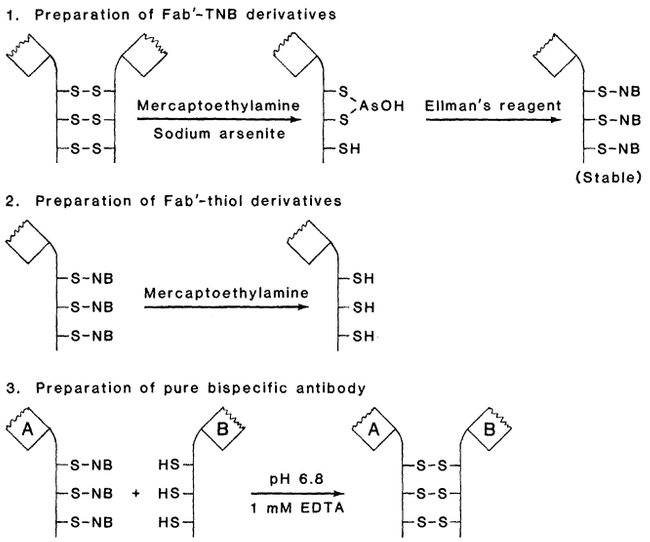

Fig. 1. Reaction sequence for the preparation of bispecific antibodies from monoclonal mouse IgG_1 fragments. Only the hinge region of the Fab′ fragments is shown.

The efficacy of sodium arsenite in preventing intrachain disulfide bonds was demonstrated by titration with Ellman's reagent. When $F(ab')_2$ was reduced in the absence of arsenite, monoclonal mouse Fab′ and rabbit Fab′ bound nearly the same amount of TNB, but mouse $F(ab')_2$ reduced in the presence of arsenite bound approximately three times more TNB. The use of Ellman's reagent (*10*) as a thiol activating agent had four important advantages. First, the Fab′-TNB derivative was a relatively stable compound, and thus a convenient intermediate, which could be stored at 4°C for several days with only slight deterioration. Second, Fab′-TNB was also a convenient source of Fab′-thiol, which could be generated by brief exposure to 2-mercaptoethylamine. Third, the reaction of a Fab′-TNB with a Fab′-thiol led to the bispecific $F(ab')_2$ as the sole product, obviating the need for separation from symmetrical dimeric products. Finally, thiol activation with Ellman's reagent allowed bispecific antibody formation in the absence of net thiol oxidation. EDTA could thus be present at all steps to prevent heavy metal–catalyzed disulfide formation, ensuring that—even without exclusion of O_2 from the atmosphere and solvents—the Fab′-thiol reactant did not self-dimerize.

An example of the formation of a bispecific antibody from an anti-avidin Fab′ and an anti-luciferase Fab′ is shown in Fig. 2, the reaction being monitored by high-performance gel exclusion chromatography. Under the coupling conditions used, the activated anti-avidin Fab′ and the thiol form of anti-luciferase Fab′ alone formed only trace amounts of $F(ab')_2$, whereas an equimolar mixture formed $F(ab')_2$ with a yield of 70 percent. (The small amount of IgG in each of the samples served as an internal chromatographic standard.) The absence of dimer

Table 1. Enzyme immobilization by bispecific antibodies. Biotinylated regenerated cellulose membranes (4.8-mm α-Metricel disks; Gelman) were prepared by CNBr activation (*16*) and substitution with biocytin (*17*). They were then incubated with or without avidin (5 μg) in 50 μl of buffer G (10 m*M* potassium phosphate and 0.6*M* NaCl; *p*H 7.4) containing bovine serum albumin (10 mg/ml) for 1 hour at 4°C. After brief washing with buffer G containing 0.1 percent Tween 20, the membranes were incubated for 2 hours at 4°C in 100 μl of buffer G containing 1 percent bovine serum albumin and the bispecific antibodies were directed against different epitopes of *E. coli* β-galactosidase (βG). After being washed five times with 0.5 ml of buffer G containing 0.1 percent Tween 20, the membranes were assayed for βG activity (*18*) or horseradish peroxidase (HRP) activity (*19*) with *o*-nitrophenylgalactoside or *o*-phenylenediamine, respectively, as chromogenic substrates, by measuring absorbance at 420 or 490 nm.

Incubation mixture	Enzyme measured	Enzyme activity immobilized
Experiment 1		
Complete*	βG	1.098†
Without avidin	βG	0.015
Without anti–avidin:βG $F(ab')_2$	βG	0.030
Experiment 2		
Complete‡	HRP	3.0§
Without avidin	HRP	0.083
Without anti–avidin:HRP $F(ab')_2$	HRP	0.114
Experiment 3		
Complete‖	HRP	0.961¶
Without avidin	HRP	0.082
Without anti–avidin:βG $F(ab')_2$	HRP	0.167
Without βG	HRP	0.107
Without anti–βG:HRP $F(ab')_2$	HRP	0.080

*Biotinylated α-Metricel disk, avidin (5 μg), anti–avidin:βG $F(ab')_2$ (0.5 μg), and *E. coli* βG (0.5 μg). †Twenty percent of βG input. ‡Biotinylated α-Metricel disk, avidin (5 μg), anti–avidin:HRP $F(ab')_2$ (0.5 μg), and HRP (1 μg). §One percent of peroxidase input. ‖Biotinylated α-Metricel disk, avidin (5 μg), anti–avidin:βG $F(ab')_2$ (0.5 μg), βG (0.5 μg), anti–βG:HRP $F(ab')_2$ (0.5 μg), and HRP (1 μg). ¶Two-tenths of 1 percent of peroxidase input.

formation when either of the reaction partners was absent suggested that the dimer formed in the complete mixture represented exclusively pure bispecific antibody. Pure product can thus be conveniently obtained by subjecting the reaction mixture to high-performance gel exclusion chromatography and collecting the $F(ab')_2$ fraction (*11*). The yields of $F(ab')_2$ obtained with many combinations of different Fab′ pieces were generally 50 to 70 percent. No attempt was made to optimize yield by modifying the reaction conditions.

Besides yield, an important consideration in evaluating our synthetic procedure is product quality. To determine whether the chemical manipulations caused irreversible alterations in the antigen-binding site, we carried a monoclonal anti–β-galactosidase $F(ab')_2$ through the procedure, thus presumably regenerating the starting material. We compared the immunoreactivity of the original and reconstituted $F(ab')_2$ in an enzyme-linked immunoassay against β-galactosidase and found no significant difference. This suggested that there was no substantial alteration or loss of antigen-binding sites. On the other hand, titration with [^{14}C]*N*-ethylmaleimide occasionally showed the presence of 0.1 to 0.2 mol of residual-free thiol per mole of $F(ab')_2$, indicating that not all disulfides were completely reformed. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed that about 10 percent of the product migrated slightly more rapidly than $F(ab')_2$, accompanied by a corresponding amount of free light chains. These observations suggested that our procedure led to some reduction and incomplete reoxidation of the disulfide bond between heavy and light chains, without significantly affecting the integrity of the antigen-binding site.

To ascertain whether the reaction products were indeed bispecific antibodies, we carried out our procedure with monoclonal Fab′ against avidin on the one hand and against *Escherichia coli* β-galactosidase or horseradish peroxidase on the other. We postulated that the resulting bispecific antibodies would be able to act as linkers for immobilization of the enzymes on a biotin-substituted matrix in the presence of avidin. As shown in Table 1, the avidin-dependent binding of β-galactosidase and peroxidase to biotinylated regenerated cellulose membranes occurred in the presence of the corresponding bispecific $F(ab')_2$. The ability of bispecific antibodies to act as heterobifunctional protein cross-linkers was even more strikingly illustrated by the immobilization of peroxidase on biotin-substituted cellulose in the presence of two bispecific antibodies, one with binding sites for avidin and β-galactosidase, the other for β-galactosidase and peroxidase, as well as both avidin and β-galactosidase. This illustrates the potential use of bispecific antibodies for the highly specific coimmobilization of multiple enzymes (*12*). Such complexes are self-assembling and functional enzyme sequences can be generated, even from impure enzyme mixtures.

In conclusion, we have described a rapid and efficient method for the preparation of bispecific antibodies from monoclonal mouse IgG_1. It differs from the cell fusion approach (*4*) by producing $F(ab')_2$ instead of intact IgG but gives pure products in higher yields with greater ease. Whereas cell fusion is labor-intensive and may not always succeed with the hybridoma pair of choice, our chemical procedure allows a single operator to prepare many different bispecific antibodies in less than 1 week, the only experimental limitation being the susceptibility of the monoclonal antibodies to selective cleavage by pepsin. Most important, many applications of bispecific antibodies require pure preparations (*12*), which are obtained directly by our chemical reconstitution method. In contrast, the complex antibody mixtures produced by hybrid hybridomas must be purified either by difficult chromatographic procedures or by immunoaffinity fractionation involving drastic desorption conditions that are likely to cause some irreversible denaturation.

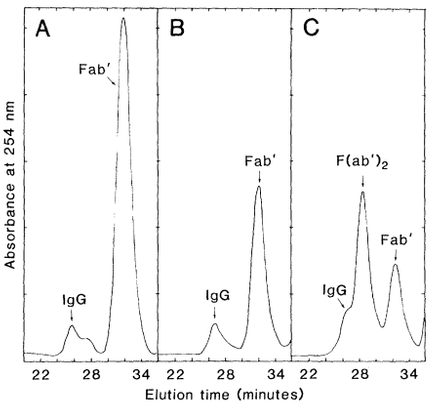

Fig. 2. Formation of bispecific antibodies. BALB/c mice were immunized by repeated injections of avidin or *Photinus* luciferase and their spleen cells were fused by conventional methods (*13*) with P3-NS1/1-Ag4 myeloma cells obtained from the Cell Distribution Center, Salk Institute. Hybrid cells were selected in hypoxanthine, aminopterin, and thymidine medium (*14*) and screened by enzyme-linked immunoassay, and selected colonies were cloned by limiting dilution on thymocyte feeder layers. Hybridomas secreting IgG_1 were grown as ascites tumors. The IgG_1 in the fluid was purified by salting out and DEAE chromatography and digested with pepsin as described in the text. The $F(ab')_2$ fragments were purified by high-performance liquid chromatography (HPLC) on a TSK-3000SW column in 0.1*M* sodium phosphate (*p*H 6.8) and then reduced overnight (3 mg/ml; 25°C) with 1 m*M* 2-mercaptoethylamine in the same buffer with 1 m*M* EDTA and 10 m*M* sodium arsenite. Solid Ellman's reagent was added to a concentration of 5 m*M* and, after 3 hours at 25°C, the excess reagent was removed by centrifugal gel filtration on Sephadex G-25 (*15*). The thiol form of the anti-luciferase Fab′ was regenerated by a 30-minute treatment with 10 m*M* mercaptoethylamine followed by centrifugal gel filtration. Although the reactions described were nearly quantitative, the yield at this stage was about 75 percent because of mechanical losses during the gel filtration procedures. Shown are (A) the TNB derivative of anti-avidin Fab′(0.19 mg), (B) the thiol form of anti-luciferase Fab′ (0.19 mg), and (C) a mixture of these, at a protein concentration of 2.4 mg/ml, incubated for 16 hours at 25°C in 0.1*M* sodium phosphate (*p*H 6.8) and 1 m*M* EDTA, all treated with 5 m*M* Ellman's reagent to dissociate any noncovalent aggregates and subjected to HPLC as described above. The starting materials showed no change during the incubation period, whereas their mixture was converted to $F(ab')_2$ with a yield of about 70 percent. Bispecific antibody was obtained with a yield of 48 percent after purification by HPLC.

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