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MODEL IMMUNE COMPLEXES FORMED BETWEEN MONOCLONAL ANTIBODIES AND A NOVEL BIVALENT AFFINITY LABEL

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SUMMARY: Murine monoclonal anti-dinitrophenyl antibodies were produced by lymphocyte hybridomas and incubated with a new bivalent affinity label, bis-(dinitrofluorobenzene)-pimelic acid amide. Stable dimers were isolated from the resulting mixture of immune complexes in high purity. Experiments were performed to show that the immune complexes are covalently cross-linked through antibody active sites. Model dimers were also formed with varying ratios of monoclonal anti-hapten antibodies based on immunoglobulin isotype. The potential uses of these complexes for analyses of the biological activites of immune aggregates are discussed.

Since it is becoming increasingly clear that antibody aggregation processes and/or immune complexes are important activators of several immune effector systems (reviewed in refs. 1,2), it is important to understand the nature of those complexes as they relate to complement (C')* components, cell surface Fc-receptors and immunoglobulin (Ig) transport and metabolic pathways. Three major problems are faced in performing such studies. First, naturally-occuring immune complexes as well as those formed with macromolecular antigens under laboratory-defined conditions are non-covalent aggregates whose physical states are unstable to experimental manipulations. Second, precise physicochemical studies of Ig structures involved in the biological activities of immune aggregates are hampered by physicochemical properties of native, multimeric antigens. Finally, the heterogeneity inherent with polyclonal antibody populations makes difficult the separate evaluations of roles played by Ig classes, subclasses (isotypes), specificities or affinities in the structure and biological potency of immune complexes.

The first problem, and to some extent the second, can be resolved through the use of bivalent affinity labels which covalently cross-link antibody molecules.

* ABBREVIATIONS USED: bis(DNFB)- , bis (dinitroflurobenzene)-; C', complement; DNFA, dinitrofluoroaniline; DNP, dinitrophenyl; Ig, immunoglobulin(s); SDS, sodium dodecyl sulfate.

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Two valuable bis-dinitrophenyl (bis-DNP) labeling reagents have been used in studies of immune complex catabolism (3), the binding of immune aggregates by macrophage Fc-receptors (4) and the interactions between immune aggregates and C'(5) including the first C' component, Clq (6). However, these reagents suffer disadvantages in that the immune aggregates formed are somewhat unstable or do not contain epitope covalently attached to antibody active sites after covalent cross-linkage.

The final problem faced in structure:function studies of immune complexes, i.e. that of antibody heterogeneity, can be resolved by using monoclonal antibodies produced by lymphocyte hybrids derived by fusing individual antibody-forming cells with myeloma cells (7). All antibody molecules produced by hybrid clones have identical antigen-binding affinity and specificity and are of the same Ig class, isotype and, presumably, idiotype (8).

In this report is presented data on the formation of stable model immune complexes consisting of well-characterized monoclonal antibodies and a novel bivalent affinity label the antigenic portion of which remains in antibody active sites after covalent modification.

MATERIALS AND METHODS

Reagents The bis-(DNFB) amides of pimelic acid were synthesized by mixing (1:2:3 molar ratio) heptanedecanoic acid, 2,4-dinitrofluoroaniline and dicyclohexyl carbodiimide (Aldrich) in K_2CO_3-dried ethyl acetate (1.0 ml/mmol DNFA) for 24 hrs in the dark. Dicyclohexylurea was filtered and the solution was rotary evaporated to an oily residue which was solubilized in minimal chloroform and recrystalized twice from methanol. Hydrolysis in 6N HCl showed the product (mp 83°) release 1.93 moles DNB/mole compound. A radioactive analog was synthesized to a specific activity of 51.7 uCi/mmol using 1,7[^{14}C]-pimelic acid (5 mCi/mmol, ICN Pharmaceuticals). The scintillation cocktail used was 'Aquasol-2' from NEN.

DNP-dextran was synthesized by forming a mixed anhydride of DNP-glycine (100 mg in 2.0 ml anhydrous dimethylformamide and 0.2 ml ethylenediamine) and ethyl chloroformate (0.3 ml) combined immediately with 2.0 g dextran T-70 (Pharmacia) in alkaline pyridine (0.1M $NaHCO_3$ 1:1 with pyridine). After rapid stirring for 60 min, 9.0 ml 0.2% formic acid was added and the mixture was dialyzed against 0.2% formate followed by dialysis against 0.1M potassium phosphate, pH 8.0. DNP conjugated Limulus polyphemus hemocyanin (DNP-LPH) was prepared as previously described (9).

The α-amino group of ε-2,4-DNP-lysine was radioiodinated by mixing 10 mmol ligand with 1.0 mCi[^{125}I]-Bolton-Hunter reagent (NEN) in 0.1M borate pH 8.5 for 60 min at 4°. The product chromatographed on thin layer silica gel plates in water saturated methyl ethyl ketone (R_f=0.69) had a specific activity of 0.13 mCi/ug. Monoclonal antibodies were radioiodinated by a modified ICl procedure (10).

Immune aggregates were prepared by incubating monoclonal IgG (30 uM) with bis-hapten reagent (150 uM in dimethylformamide) in a 1:20 (v/v) ratio for 2 hrs at

room temperature in the dark and in 0.1M phosphate pH 8.0. Reactions, terminated by the addition of 0.1 volume of 0.2M glycine-OH, pH 7.0 buffer, were applied to 5 x 100 cm Ultrogel AcA22 molecular sieve columns in 0.1M borate, pH 8.0.

Animals, Cell Hybrids and Monoclonal Antibodies BALB/c mice (Cumberland Farms) were vaccinated with DNP_{17}-LPH (17 moles DNP/mole LPH) or DNP_3-dextran in complete Freund's adjuvant (3:1 adjuvant:antigen, ref. 11) intrascapularly on days 0,14 and 21. Spleen cells were harvested from individual animals on day 7 (primary immunocytes), 17 (secondary immunocytes) or 24 (hyperimmune cells).

The lymphocyte fusion procedure was essentially that of Galfre et al. (12) modified to include the use of the SP2/0-Ag14 (13) or P3-X63-Ag8.653 (14) cell lines. Fusion was accomplished by mixing 10^7 myeloma cells with 10^8 splenocytes in 40% polyethylene glycol 1540 (J.T.Baker), and, after selection in HAT media, cloning was accomplished using BALB/3T3 cell (American Type Culture Collection) conditioned medium and 0.2% SEACHEM agarose (med. endosmosis) in 64-well culture cluster dishes (Costar).

Clones secreting anti-DNP antibodies were detected prior to and after subcloning by analyzing culture medium supernates in a Farr-type radioimmune assay (15) with [^{125}I]-DNP-lysine and murine anti-DNP ascitic fluid prepared as described in (16). Specific antibody-producing clones were grown as ascitic hybridomas in 'Pristane' (Aldrich) primed mice (0.1 ml reagent injected intraperitoneally 7 days prior to cell inoculation). Anti-DNP antibodies were purified from resulting ascitic fluids by affinity chromatography (16) and characterized by radial immunodiffusion assay using rabbit antisera specific for murine myeloma heavy and light chains specified in Table I. Binding affinities for DNP were determined by equilibrium dialysis (17)

RESULTS

Table I shows the molecular characteristics of the products of 8 hybridomas obtained from various fusions of anti-DNP immune lymphocytes with the non-secreting,

Table I. Characteristics of Purified Monoclonal Anti-DNP Antibodies and Myeloma Proteins.

Protein	Source	H-chain	L-chain	K_o(x$10^6$$M^{-1}$)	n
DNP-1	primary anti-DNP-dextran immunocyte hybrid	μ	κ	29.2	10
DNP-2	primary anti-DNP-LPH immunocyte hybrid	γ2a	κ	17.2	2
DNP-3	secondary anti-DNP-LPH immunocyte hybrid	μ	n.s.r.	30.1	10
DNP-4	" " " "	γ2a	κ	34.5	2
DNP-5	hyperimmune anti-DNP-LPH immunocyte hybrid	γ2b	κ	15.7	2
DNP-6	" " " "	μ	κ	0.8	5
DNP-7	hyperimmune anti-DNP-dextran immunocyte hybrid	γ1	n.s.r.	29.8	2
DNP-8	" " " "	μ		63.3	10
21	MOPC-21 myeloma tumor	γ1	κ	-	
LPC-1	LPC-1 myeloma tumor	γ2a	κ	-	
J606	J606 myeloma tumor	γ3	κ	-	
195	MOPC-195 myeloma tumor	γ2b	κ	-	
104E	MOPC-104E myeloma tumor	μ	λ1	-	
315	MOPC-315 myeloma tumor	α	λ2	9.2	2

K_o (aver.intrinsic assoc. constant) = reciprocal moles ligand bound/mole antibody when half of the available antigen-binding sites (n)are filled. n= extrapolated abcissa intercept value used. n.s.r.= not strongly reactive with rabbit anti-MOPC-46B (mouse κ-type light chain) antiserum in immunodiffusion assay (15). The literature K_o value for protein 315 is 1.6 x 10^7 M^{-1} (17) using the same equilibrium dialysis techniques.

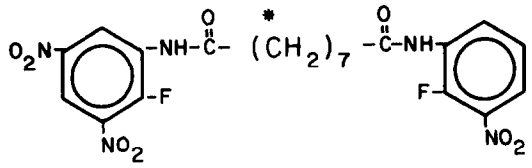

Figure 1. Bis(dinitrofluorobenzene) Pimelic Acid Amide. The asterisk (*) represents the 1,7[^{14}C] -analog.

8-azaguanine-resistant myeloma cells. After antibodies were purified from tumor ascitic fluids, clones 2 and 5 were found to produce IgG2a and IgG2b isotypes with approximately similar binding affinities for the DNP ligand. Thus, these two products were used to form model immune complexes with radioactive bis-(DNFB)-pimelic acid amide (Figure 1). The complexes so formed are displayed on sodium dodecyl sulfate (SDS, Bio-Rad) polyacrylamide gels (Figure 3) to consist of dimers, trimers and larger polymers which can be isolated by molecular sieve chromatography (Figure 2).

Table II shows that, by increasing the concentration of [^{14}C]-bis hapten during affinity labeling, there is a corresponding incremental increase in the specific

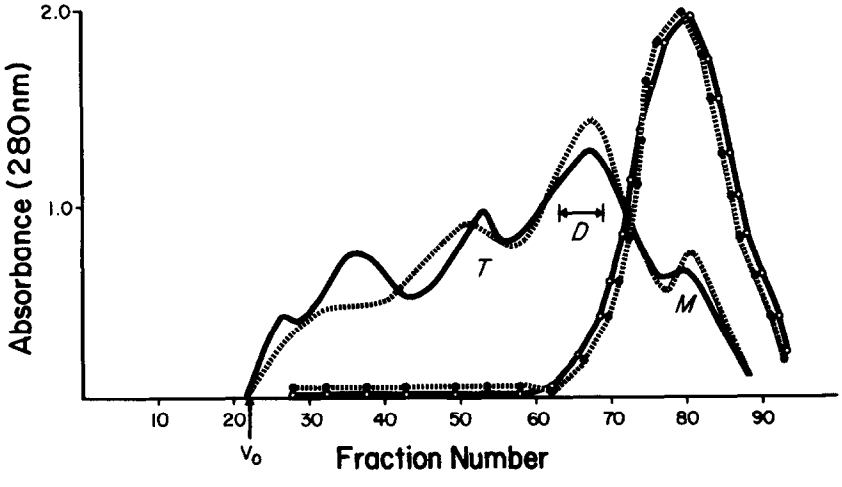

Figure 2. Molecular Sieve Chromatograms of Monoclonol Antibody: Bis(DNFB)pimelate Amide Reaction Mixtures.

Ultrogel AcA22 elution profiles of protein DNP-2 (IgG2a anti-DNP, solid lines) and protein DNP-5 (IgG2b anti-DNP, dashed lines) incubated with bis affinity label in the absence and presence (lines with circles) of 10 mM N-DNP-glycine. V_o= column void volume as measured with blue dextran.

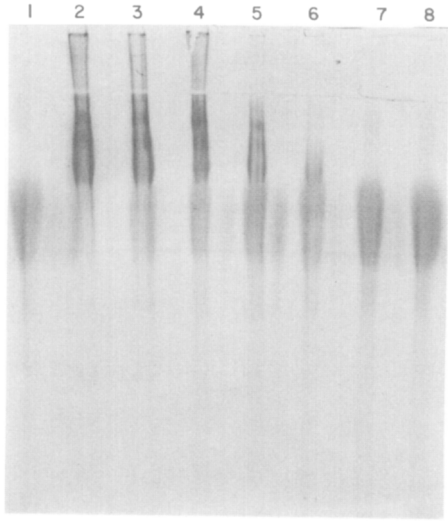

Figure 3. SDS-polyacrylamide Gel Electrophoretogram of Monoclonal IgG Antibodies Cross-linked with Bis(DNFB) Pimelic Acid Amide.

Gels (3% stacking, 5% resolving) were prerun 120 min to remove persulfate. Protein (25 - 50 ug) was applied to individual lanes and electrophoresis was performed in 0.1% sodium dodecyl sulfate (Bio-rad) for 4 hrs, 30 mA then stained with 0.05% Coomassie G250 in 3% $HClO_4$ and destained in distilled water.
LANE (1) protein DNP-2 ; (2) reaction mixture of DNP-2 + bis affinity label;
(3) - (8) same as Lane 2 except for the simultaneous incubation of 150 uM bis affinity label and 1,10,50, 100, 500 and 1000 uM N-DNP-glycine.

Table II. Incremental Changes in Bivalent Affinity Label Cross-linked IgG2a Anti-DNP Monoclonal Antibodies (protein DNP-2).

[^{14}C]-bis affinity label (cpm/umol)	Concentration of bis affinity label in reaction (uM)	Specific activity of isolated dimers (cpm/umol IgG)
5,300	0.06	759
10,100	0.06	1,660
21,100	0.06	4,445
42,600	0.06	10,766
42,600	0.60	21,732
42.600	1.20	8,153
42,600	2.40	3,307
42,600	4.80	1,050

Dimers were isolated chromatographically as indicated in Figure 2.

activity of isolated dimers even though labeling was performed at half-saturation (Ka) ligand concentrations. This result is confirmed with unlabeled bis-hapten when the resulting complexes are analyzed on SDS gels (Figure 3). Conversely, at a fixed amount of [^{14}C]-bis(DNFB) pimelate amide, the specific activity of cross-linked dimers is progressively decreased (Table II). The formation of aggregates was also progressively inhibited by the addition of DNP-glycine during affinity labeling (Fig.3), and aggregates do not form with myeloma proteins except for MOPC-315, an IgA myeloma immunoglobulin with polynitrophenyl ligand-binding properties (18). Finally, immune complexes formed with bis-(DNFB)-pimelic acid amide do not dissolve in 10 mM DNP-glycine (not shown). These results suggest that the monoclonal antibody dimers, trimers and larger polymers consist of Ig molecules which are cross-linked covalently through their antigen-binding sites.

Experiments to establish the feasibility of intentionally designing model immune complexes consisting of varying ratios of IgG2a and IgG2b antibodies were performed by carrying out cross-linkage reactions in 3-fold Ka concentrations of bis-hapten reagent and 1:1 or 2:1 ratios of [^{125}I]IgG2a:[^{131}I]IgG2b antibodies. Dimers formed under these conditions were isolated chromatographically and evaluated for radioiodine contents. Results (Table III) show that ratios of about 1:1 and 2:1 are maintained after covalent cross-linkage. To further understand the molecular compositions of thes hybrid complexes, dimers were incubated with

Table III. Composition of Monoclonal IgG Dimers Formed with Bis(DNFB)-Pimelate Amide.

[^{125}I]-IgG2a / [^{131}I]-IgG2b (cpm x 10)	in cross-linkage rxn. ratio	in isolated dimers (cpm x 10)	ratio	in supernatant after adsorption with anti-γ2a-cellulose (cpm)	adsorbed radiolabel %
57.4 / 59.3	0.97	13.4 / 14.4	0.93	56 / 6,325	72
65.2 / 30.6	2.13	16.3 / 7.9	2.06	81 / 3,004	91

Specific activities for monoclonal anti-DNP antibodies were 12,756 cpm/ug and 13,555 cpm/ug for protein DNP-2 (IgG2a) and DNP-5(IgG2b) respectively. IgG concentrations were estimated using $\epsilon^{1\%}_{1cm}$= 15 at 278 nm. Rabbit antiγ-2a (H-chain)-specific cellulose immunoadsorbant (0.1 ml packed vol. and capable of removing 4.3 ug IgG2a from solution) was incubated with 2 ug/ml of dimer complexes at 4° for 30 min, pelleted and the supernatant analyzed. A second adsorption removed no further radiolabel from the dimer solution.

cellulose-immobilized rabbit anti-murine γ2a or anti-murine γ2b serum (prepared as described in 19), and supernates were again evaluated for radiolabel content. Results (Table III) confirm and extend the initial observations to indicate that the 1:1 mixture consists of a random distribution of 1:2:1 (2a2a:2a2b:2b2b) while the 2:1 mixture consists of about a 3:2:1 ratio.

DISCUSSION

Using monoclonal antibodies, elusive immunochemical questions of the roles played by antibody specificity, affinity and isotype in immunoglobulin effector functions can be investigated. One approach reported here is to prepare model immune complexes in the form of stable antibody dimers cross-linked with a new class of bivalent affinity labeling reagent, the bis-(DNFB)-amides of dicarboxylic acids. Detailed studies of complexes formed with a series of these reagents and their photoactivatable analogs will be described elsewhere. Here, based on previous results (3,6), the pimelic acid (7-carbon spaced) bis-hapten was synthesized and used to produce IgG dimers. The facts that (a) the affinity label does not cross-link myeloma proteins which are not specific for the DNP hapten, (b) there is an incremental increase in the specific activities of aggregates formed on incubation of antibodies with low concentrations of [^{14}C]-bis-hapten (Table II), (c) resulting aggregates are mostly insoluble in the presence of high concentrations of mono-hapten or during electrophoresis in SDS polyacrylamide gels (Figs 2 & 3) and (d) affinity label cross-linking is inhibited by incremental increases in mono-hapten (Table II, Fig. 2), all suggest that the dimers are covalently cross-linked through Ig antigen-binding sites.

When dimers are formed by mixing equal amounts of radiolabeled IgG2a and IgG2b antibodies with bis-hapten reagent, they consist of a random 1:2:1 mixture (2a2a: 2a2b:2b2b). This ratio changes to 3:2:1 when initial reactions contain twice the amount of IgG2a as IgG2b antibodies (Table III). Such results allow the speculation that one should be able to generate model immune complexes of any desired composition by varying the concentration, class, isotype, affinity and specificity of monoclonal antibodies used. With such complexes it may now be possible to

standardize immune complex assays and compare the activities of native immune aggregates in biologically meaningful ways.

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