Production of Bifunctional Monoclonal Antibodies to Human IgG and Horseradish Peroxidase and Their Utilization for Testing Anti-HIV Antibodies

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Bispecific antibodies are purified from ascitic fluid by successive affinity chromatography on HRP-Sepharose and IgG-Sepharose. The ability of the affinity purified bispecific antibodies to bind human IgG and horseradish peroxidase is demonstrated in the immunoenzyme assay (identification of anti-HIV antibodies.

Key Words: *quadroma; bispecific antibodies; human IgG; peroxidase; anti-HIV antibodies*

Hybridoma technique allows for the production of both mono- and bispecific monoclonal antibodies (MAb). A bispecific monoclonal antibody (BMAb) carries binding sites for two different antigens. In order to obtain such antibodies, two hybridomas producing different MAb are fused. MAb of both parental types, BMAb, and inactive immunoglobulins are formed as a result of recombination of the light and heavy chains of parental immunoglobulins in hybrid hybridomas (quadromas) [2]. Bispecific monoclonal antibodies may be used in immunoenzyme analysis. In this work we studied the properties of BMAb to human IgG and horseradish peroxidase (HRP), which were produced by a murine quadroma. We also describe the technique whereby a BMAb-secreting quadroma was obtained, the isolation of BMAb, and their use in the testing of anti-HIV antibodies.

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MATERIALS AND METHODS

Human IgG was isolated from donor plasma by chromatography on protein A-Sepharose (Pharmacia) according to the manufacturer's recommendations. Hybridomas secreting anti-human BMAb were produced as described [3] by fusing splenocytes derived from mice immunized with human IgG with X63-AG8.653 myeloma cells. Producer clones were identified by immunoenzyme and radioimmunological assays. Clone HIG-20 secreting antibodies (IgG_1) to the Fc fragment of human IgG was used. Production of hybridoma $36F_9$ secreting anti-HRP MAb (murine IgG_1) was described elsewhere [1,4]. This hybridoma carries two selective markers: it is sensitive to HAT medium (hypoxanthine, aminopterin, thymidine, HAT^S) and is resistant to actinomycin D (AMD^R). Quadromas were obtained as follows: 10^6 HIG-20 cells were fused with 10^6 $36F_9$ cells (AMD^R/HAT^S) as described previously [1,4] and seeded in 96-well plates. Quadroma clones were selected on HAT^S medium containing 7 μg/ml AMD. Bispecific monoclonal antibodies were tested in double-antigen ELISA, which was performed as follows. The

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plates were incubated overnight at 0°C with human IgG (1 μg/well in 100 μl Na-phosphate buffer, pH 7.5) and then incubated with culture media diluted 10-100 times in the presence of HRP (0.1 μg/100 μl). The imunoenzyme reaction was performed with ortho-phenylenediamine after a 1-h incubation and washing. Ascitic fluid of BMAb-secreting quadromas was obtained as described elsewhere [1,4]. Bispecific MAb were isolated from the ascitic fluid by successive affinity chromatography on HRP-Sepharose and IgG-Sepharose columns. Horseradish peroxidase (Sigma) and human IgG (Pharmacia) were conjugated with BrCN-Sepharose (Pharmacia) according to the manufacturer's recommendations. At the first step, ascitic fluid was centrifuged (15,000 g, 0°C, 1 h), diluted 10-fold with Na-phosphate buffer with 0.15 M NaCl, and loaded on an HRP-Sepharose column. The isolation procedure was described in detail elsewhere [1,4]. Affinity purified antibodies (anti-HRP antibodies and BMAb) were further loaded on an IgG-Sepharose column. Eluates from this column contained anti-IgG/HRP BMAb. The antibody concentration was determined spectrophotometrically, assuming that A_{280}=1.35 corresponds to an antibody concentration of 1 mg/ml. Anti-HIV antibodies were identified in the sera of HIV-infected patients and healthy donors by the immunoenzyme method, using BMAb and Skrin-VICH [HIV-screen] kits (A/O D. Mazai, Russia). Immune plates with recombinant HIV-1 and HIV-2 proteins (p18, p24, gp41, gp120, gp38, and gp110) were successively incubated with test sera and anti-human IgG MAb/HRP conjugates or with BMAb (0.15 μg/ml) mixed with HRP (0.75 or 1.5 μg/ml) in the kit buffer. The reaction with ortho-phenylenediamine was performed after 1-h incubation at 37°C and read at 472 nm. Incubation and washing were carried out according to the manufacturer's recommendations. Sera of HIV-infected individuals were obtained from the Central Institute of Epidemiology. The presence of anti-HIV antibodies was confirmed by immunoblotting.

RESULTS

Twenty-eight actively growing quadroma clones were obtained after fusing HIG-20 and $36F_9$ hybridomas. BMAb-secreting clones were identified by immunoenzyme assay by the ability of antibodies to bind two antigens (IgG and HRP) simultaneously. Fifteen clones manifested this ability. Quadromas with the most potent positive reponse in this test were cloned 2 more times. About 95% BMAb-secreting clones were obtained after the second cloning.

TABLE 1. Absorbance Recorded in HIV–Positive and HIV–Negative Sera Using a Skrin–VICH Kit and Anti–IgG/HRP BMAb

№ of HIV-positive serum	Anti-IgG antibodies conjugated with HRP (kit component)	Bifunctional antibodies	
		5-fold excess of HRP	10-fold excess of HRP
1	0.237	0.227	0.289
2	0.267	0.269	0.302
3	0.265	0.245	0.299
4	0.242	0.217	0.296
5	0.418	0.341	0.492
6	0.264	0.233	0.298
7	0.450	0.379	0.522
8	0.677	0.578	0.831
9	0.331	0.332	0.413
10	0.433	0.394	0.515
11	1.044	0.816	1.209
12	1.619	1.410	1.899
13	1.725	1.413	1.946
14	1.888	1.646	2.119
15	1.472	1.271	1.732
16	2.109	1.792	2.237
17	1.290	1.059	1.623
18	1.972	1.694	2.236
19	2.231	2.008	2.207
20	1.549	1.183	1.674
HIV–negative sera ($M \pm s$)	0.117±0.014	0.105±0.014*	0.123±0.017

Note. Asterisk indicates values statistically different from the conjugate at $p<0.001$.

Ascitic fluid obtained from the clone $90C_{10}$ was used to purify BMAb. It contained 4 antibodies: BMAb, anti-IgG antibodies, anti-HRP antibodies, and inactive antibodies. Inactive antibodies were produced due to the association of heterologous light and heavy chains of parental immunoglobulins. Bispecific MAb were isolated by successive chromatography on HRP-Sepharose and IgG-Sepharose. The concentration of BMAb in the ascitic fluid was 2.93±0.51 mg/ml, which is comparable to the MAb yield in some other hybridomas. The concentration of anti-HRP MAb was 2.74±0.38 mg/ml, while the concentrations of anti-human IgG Ab and inactive antibodies were 2.52±0.32 and 5.26±0.47 mg/ml, respectively ($M \pm \sigma$, three separate fractionations of ascitic fluid pooled from 3 mice).

Theoretically, anti-human IgG/HRP BMAb are a convenient tool for probing human antibodies in a one-step peroxidase-antiperoxidase (PAP) tech-

TABLE 2. Titers of HIV-Positive Sera in Which Absorbance Is Equal to 1, Using a Skrin-VICH Kit and anti-IgG/HRP BMAb

№ of HIV-positive serum	Anti-IgG antibodies conjugated with HRP (kit component)	Bifunctional antibodies	
		5-fold excess of HRP	10-fold excess of HRP
1	1:3000	1:2200	1:4000
2	1:8100	1:6000	1:13400
3*	1:9600	1:5400	1:11800
4	1:18000	1:12000	1:24300
	1:6000	1:4400	1:9700
7	1:13300	1:9400	1:24300
9	1:54200	1:42200	1:80800
10*	1:6300	1:4000	1:7700

Note. Asterisk indicates sera obtained from HIV-2-infected individuals.

nique. The efficiency of BMAb was tested in immunoenzyme analysis of human sera for the presence of anti-HIV antibodies, using Skrin-VICH plates with immobilized HIV-1 and HIV-2 recombinant proteins. For this purpose 53 HIV-positive and 38 HIV-negative sera were analyzed for the presence of anti-HIV antibodies by three techniques: 1) all the components of the Skrin-VICH kit and anti-human IgG conjugated with HRP; 2) the conjugate was replaced with BMAb: the antibody (0.15 μg/ml) was added with a 5-fold excess of HRP (0.75 μg/ml); 3) BMAb was added with a 10-fold excess of HRP (1.5 μg/ml). Anti-HIV antibodies were identified in all 53 HIV-positive sera and all 38 HIV-negative sera proved to be negative. The absorbance values of the relatively weakly HIV-positive sera (<2.3 in any of these tests) are given in Table 1. When BMAb were used with 10-fold excess of HRP the absorbance of all HIV-positive sera was higher compared with that recorded when the conjugate was added. When BMAb were added with a 5-fold excess of HRP, the absorbance was lower compared with that read in the conjugate reaction; however, anti-HIV antibodies were readily identified. It should be mentioned that when BMAb were added at a 5-fold excess of HRP, the mean absorbance of HIV-negative sera was significantly lower than that observed after the addition of conjugate (Table 1).

In the second series 10 HIV-positive sera were analyzed, which were randomly selected from the 33 sera whose absorbance in each test was >2.3. Eight sera were obtained from HIV-1-infected persons and 2 sera were from HIV-2-infected persons (Table 2). Each of these 10 sera was titrated, and the titer yielding an absorbance equal to 1 was determined (Table 2). The highest efficiency was reached when BMAb were added with a 10-fold excess of HRP. In this analysis, for each serum the titer yielding an absorbance equal to 1 was higher than that obtained in the reaction with the conjugate or BMAb with a 5-fold excess of HRP.

Thus, we generated quadromas that produce BMAb with a bispecificity for human IgG and HRP. When employed at a 5- or 10-fold excess of HRP, these MAb enabled us to effectively identify anti-HIV antibodies in HIV-positive sera.

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