AN IMPROVED METHOD FOR THE PRODUCTION OF ANTISERA TO G1m ALLOTYPES

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Summary An improved method is described for the production of rabbit anti-Gm sera using IgG subclasses isolated by pH gradient elution from protein A-Sepharose. The production rates of anti-G1m(x) and anti-G1m(f) were increased by immunization with Fc fragments of IgG1(a,x) and F(ab')₂ fragments of IgG1(a,f), respectively.

INTRODUCTION

As a first step to promote the routine use of Gm-typing in forensic and genetic studies, a method has recently been reported for preparing rabbit anti-Gm sera using purified normal IgG subclasses as immunogens (Tamaki *et al.*, 1981). These heteroantisera—anti-G1m(x), anti-G1m(f), anti-G3m(b¹), anti-G3m(t), and anti-G3m(g)—can distinguish the 9 Gm phenotypes in the Japanese population. The method appeared easily accessible to all interested workers as it utilized normal IgG instead of myeloma proteins. However, the problem of raising the production rates of anti-G1m(x) and anti-G1m(f) remained to be solved. The present paper briefly reports an improvement on the previous method.

MATERIALS AND METHODS

Isolation of IgG. Human plasma unsuitable for clinical use was obtained from a Japan Red Cross blood center. Sera were separated and Gm-typed by the conventional hemagglutination inhibition technique using anti-D-coated group O cells and specific anti-Gm sera. The anti-D, anti-Gm sera, and a panel of standard Gm sera were generously provided by Prof. H. Matsumoto. Rabbit anti-Gm sera produced in our laboratory were also used. IgG of appropriate Gm type was isolated by ammonium sulfate precipitation at 35% saturation followed by chromatography on a DE-52 (Whatman) column. The eluate obtained with 0.01 M phosphate buffer (PB), pH 7.6, was concentrated by ultrafiltration, dialyzed against distilled water,

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lyophilized, and stored at 4°C until use. Its purity was checked by immunoelectrophoresis using anti-human serum (CAPPEL) and anti-IgG (produced in our laboratory).

Isolation of IgG subclasses. IgG subclasses were isolated by the method of Duhamel *et al.* (1979). Two hundred milligrams of IgG in 10 ml of 0.15 M citrate-phosphate buffer (CPB), pH 7.0, were applied to a 1.5×10 cm column of protein A-Sepharose CL-4B (Pharmacia). The effluent contained pure IgG3 as demonstrable by Gm-typing. The protein A-bound IgG (hereafter referred to as IgG1-rich IgG) was eluted with a pH gradient of CPB generated by a series of 3 equivalent volume chambers containing 0.15 M CPB (pH 5.0), 0.15 M CPB (pH 4.5), and 0.1 M citric acid (pH 2.2), respectively. Two overlapping peaks were obtained, the first being enriched for IgG2 and the second for IgG1. This was confirmed by Gm-typing. In order to minimize the contamination of the subclasses by each other, only the fractions (hereafter referred to as IgG2 and IgG1, for convenience) corresponding to the first half of the first peak and the second half of the second peak were pooled and kept frozen until used.

Isolation of Fc fragments. Fc fragments were prepared by the method of Porter (1959) and Michaelsen and Natvig (1971) with slight modification. Two hundred milligrams of IgG1 of G1m(a,x) type were incubated with 2 mg papain (Sigma) in 0.1 M PB, pH 7.0, containing 0.01 M L-cysteine monohydrochloride and 0.002 M ethylenediamine tetraacetic acid disodium salt at 37°C for 30 min. The digest was dialyzed against 0.01 M PB, pH 7.6, and applied to a 1.5×10 cm DE-52 column. After washing with the same buffer, the bound Fc fragments were eluted with 0.2 M PB, pH 7.6, and stored at -20°C after concentration and sterile filtration. The purity of the Fc fragment preparation was checked by immunoelectrophoresis using specific anti-IgG, anti-Fab (Hoechst), and anti-Fc antiserum (Hoechst).

Isolation of F(ab')₂ fragments. A slight modification of the method of Hudson and Hay (1980) was used. Three milligrams of pepsin were added to 5 ml of 2% solution of IgG1 of G1m(a,f) type in 0.1 M sodium acetate buffer, pH 4.5. The mixture was incubated at 37°C for 18 hr. The peptic digest was adjusted to pH 7.0 with 1 M NaOH, dialyzed against 0.1 M PB, pH 7.0, and subjected to affinity chromatography on protein A-Sepharose CL-4B to remove the Fc' fragments and undigested IgG1. The effluent containing F(ab')₂ fragments was concentrated, filtered sterile, and kept at -20°C until use. Its purity was tested by immunoelectrophoresis.

Immunization. Anti-G1m(x) serum was produced in the light of previous work (Giesen *et al.*, 1974; Goosen *et al.*, 1981). The rabbits were immune-paralyzed with an intravenous injection of 10 mg IgG2(n) from which aggregated protein had been removed by ultracentrifugation. Three days later 1 mg Fc fragments of IgG1(a,x) mixed with Freund's complete adjuvant was given intramuscularly and subcutaneously. The rabbits were boosted once a month with Fc fragments. The intravenous administration was only repeated if no precipitating antibody against IgG

was detected. Three weeks after the first and 1 week after each booster injection, the animals were test-bled.

Immunization to G1m(f) was carried out without inducing unresponsiveness. Three milligrams of F(ab')₂ of IgG1(a,f) emulsified in Freund's complete adjuvant were administered intramuscularly and subcutaneously. One or two booster injections were given at intervals of a month. The animals were exsanguinated one week after the last injection.

Testing for antibody. The procedure used was essentially the same as that described in the preceding paper (Tamaki *et al.*, 1981). Rabbit antisera were inactivated by heating at 56°C and absorbed with a mixture of group O, A, and B red cells, and subsequently with one-third its volume of 1% IgG in saline negative for the Gm factor under study. The absorbed sera were tested for anti-Gm antibodies by the conventional hemagglutination inhibition method.

RESULTS AND DISCUSSION

Table 1 compares the results of the present and previous studies. As noted earlier, the Fc fragments of type Gm(a,x,g) IgG1-rich IgG were successfully used to produce anti-G1m(x) antibody after immunization experiments with intact molecules of IgG had ended in failure (Tamaki *et al.*, 1981). However, immunization of a total 7 rabbits with the Fc fragments over a one-year period thereafter resulted in no production of anti-G1m(x). It has been shown that IgG1 is very low immunogenic in rabbits, but that IgG1 subclass-specific antisera can be produced by immunization following induction of unresponsiveness to the other subclasses (Spiegelberg and Weigle, 1968; Giessen *et al.*, 1974; Goosen *et al.*, 1981). With this and our experience in mind, 8 rabbits were injected first with IgG2 protein to suppress the production of antibodies against the common antigens on the IgG molecule. Next, the animals were immunized with Fc fragments of IgG1(a,x) instead of IgG1-rich IgG. This was successful in 2 rabbits and appears promising.

In order to increase the production rate of anti-G1m(f) antibody, rabbits were immunized with F(ab')₂ fragments of the IgG1(a,f) subclass in place of the IgG1-rich IgG(a,f,b¹,b³) used in the previous experiments. This gave remarkably good results.

Table 1. Production of anti-G1m(x) and anti-G1m(f) sera.

Antibody sought	Immunogen (Gm type)	Production rate	Antibody titer
Anti-G1m(x)	IgG1-rich IgG(a,x,g)	0/6	—
	Fc(a,x) of IgG1-rich IgG(a,x,g) without tolerance induction	1/7	8
	Fc(a,x) of IgG1(a,x) with tolerance induction	2/8	4
Anti-G1m(f)	IgG1-rich IgG(a,f,b ¹ ,b ³)	2/6	256
	F(ab') ₂ of IgG1(a,f)	7/11	64

It is a pity, however, that the anti-G1m antibodies have low titers compared with the anti-G3m.

Since some of the anti-D sera of different Gm specificities are practically unavailable, it is necessary that an alternative Gm-typing method be developed for routine use. This requires specific antibodies with much higher titers than can be expected from the rabbit antisera so far produced. Therefore, the introduction of monoclonal antibodies may be in order. A study in that direction is in progress.

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