# TECHNICAL NOTE

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# Identification of Human Blood with Hybridoma-Derived Antibody to Human Immunoglobulin G

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**ABSTRACT:** During production of monoclonal anti-Gamma (Gm) antibody by the hybridoma technique, an antihuman immunoglobulin G (IgG) antibody was obtained. Unlike conventional antihuman IgG heteroantisera, this antibody reacted with the serum of humans and chimpanzees but did not cross-react with that of other primates or lower animal species in hemagglutination-inhibition tests with anti-D-coated red cells. To examine for the practical utility of the antihuman IgG antibody in an enzyme-linked immunosorbent assay (ELISA) for identification of human blood, microtiter wells were coated with human IgG and allowed to react with the antibody in the presence of human or animal serum under test. The bound antibody was detected with enzyme labeled antimouse IgG. The ELISA gave satisfactory results.

**KEYWORDS:** pathology and biology, immunology, immunoglobulins, species identification, bloodstains, monoclonal antibodies, enzyme-linked immunosorbent assay

Antihuman immunoglobulin G (IgG) serum is one of the antisera that are used for the identification of human bloodstains. The antiserum, usually containing antibodies cross-reactive with blood from various animal species, must be either absorbed with appropriate animal serum or diluted highly enough to make the cross-reactivity negligible. If a human-specific antiserum can be prepared by immunization and without later absorption, it is the reagent of choice.

While preparing monoclonal anti-Gamma (Gm) antibody by the hybridoma technique, we obtained an antihuman IgG antibody which could differentiate human from animal blood. Here we describe the use of the hybridoma-derived antihuman IgG antibody in an enzyme-linked immunosorbent assay (ELISA) for identification of human blood.

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## Materials and Methods

### *Preparation of Antigens*

IgG was isolated from normal human serum by ammonium sulfate precipitation and diethylaminoethyl (DEAE)-cellulose column chromatography. IgG3 was isolated from the IgG by affinity chromatography on Protein A-Sepharose (Pharmacia Fine Chemicals) as described by Hjelm [*1*]. The conventional hemagglutination-inhibition method was used for Gm typing of human IgG.

### *Preparation of Hybridoma*

A modification of the technique described by Galfre et al [*2*] was used. Male BALB/c mice were immunized by intraperitoneal injections, ten days apart, of 100 $\mu$g of IgG3 of G3m(21) type emulsified in Freund's complete adjuvant. Blood samples were taken from the ophthalmic venous plexus and tested for antihuman IgG by agar gel immunodiffusion and for anti-G3m(21) as described below. Four days after the last injection, $10^8$ spleen cells were fused with $10^7$ mouse myeloma (NS-1) cells by the use of 50% polyethylene glycol 6000 (Koch-Light Laboratories). Twenty-five millilitres of RPMI 1640 were then added slowly with gentle stirring, and the cells were spun down at 500 $\times$ *g* for 10 min. The cell pellet was mixed with $10^7$ mouse thymocytes. The mixture was suspended in 50 mL of RPMI 1640 containing 15% fetal calf serum (FCS), and aliquoted into 24-well culture plates. On the following day, 1 mL of RPMI 1640 containing 15% FCS, $10^{-4}M$ hypoxanthine, $4 \times 10^{-7}M$ aminopterine, and $1.6 \times 10^{-5}M$ thymidine (HAT medium) was added to each well. Half of the medium in each well was replaced by HAT medium every two to three days until the twelth day after fusion. Thereafter half of the medium was replaced by HT medium (HAT medium minus aminopterine) three times at intervals of three to five days.

Three weeks after fusion, cultures were screened for antibody production by hemagglutination-inhibition on a microflocculation slide.

### *Inhibition of Indirect Hemagglutination*

A drop of culture supernatant was mixed with a drop of a 1 : 10 dilution of human or animal serum on a slide. The slide was gently shaken on a rotary shaker for 10 min at room temperature. A drop of a 0.2% suspension of anti-D-coated red cells was added to the mixture. After a further 30 min the slide was observed for agglutination.

### *Solid-Phase Micro-ELISA*

Microtiter wells were coated with 10 $\mu$g/mL of human IgG dissolved in 0.05*M* sodium carbonate buffer, pH 9.6. Equal volumes of each culture supernatant and a 1 : 10 dilution of human or animal serum in 0.01*M* phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween), pH 7.4, were mixed in a test tube and incubated at room temperature for 15 min. A 100-$\mu$L aliquot of the mixture was transferred to the coated well and incubated for 15 min at room temperature. The well was washed with PBS-Tween, and 100 $\mu$L of 500-fold diluted peroxidase labeled anti-mouse IgG (Miles) was placed for 15 min. The well was washed as above, to which was added 100 $\mu$L of the substrate mixture containing 50 mg of *o*-phenylenediamine dihydrochloride and 20 $\mu$L of 30% hydrogen peroxide in 100 mL of 0.05 *M* phosphate-0.024 *M* citric acid buffer, pH 5.0. The absorbance of the colored product was measured on a MicroELISA Minireader (Dynatech) at 490 nm.

## Results and Discussion

Mouse antisera formed single precipitin lines with human IgG in agar gel immunodiffusion tests. Of the four mice immunized, two produced anti-G3m(21) antibodies. Since the present study was originally aimed at producing monoclonal anti-G3m(21) antibody, spleen cells from these two mice were fused with myeloma cells.

All culture supernatants from 48 wells strongly agglutinated anti-D-coated cells. G3m (21)-positive IgG inhibited the agglutination by culture supernatants from 47 of the 48 wells. G3m(21)-positive and no G3m(21)-negative IgG inhibited the agglutination by the supernatant from the remaining well. These results indicate that the 47 cultures produced antihuman IgG isotypic antibodies, and that the remaining one culture produced anti-G3m(21) antibody. The culture producing the anti-G3m(21) was cloned and will be described elsewhere.[2]

Compared with conventional antihuman IgG sera, hybridoma-derived antihuman IgG antibodies in some culture wells even before cloning are expected to be less heterogeneous and less interspecies-reactive. To investigate this possibility, culture supernatants were tested for cross-reactivity with the serum of various animals by the hemagglutination-inhibition technique. Of the 47 wells examined, 40 contained antihuman IgG antibodies whose reactions with anti-D-coated cells were inhibited by Japanese monkey serum. The other seven contained the antihuman IgG that reacted only with the serum of humans and chimpanzees (Table 1). Since the seven cultures showed the same antibody specificity, their supernatants were pooled and tested for use in ELISA. As in the hemagglutination-inhibition test, human as well as chimpanzee serum and no other serum inhibited the reaction of the antihuman IgG with the human IgG coat on the solid phase (Table 2).

TABLE 1—*Inhibition of agglutination by various sera.*[a]

| Well | Man | Chimp | Macaque[b] | Baboon | Dog | Bull | Hog | Rat | Rabbit | Snake |
|---|---|---|---|---|---|---|---|---|---|---|
| 31A-5 | − | − | + | + | + | + | + | + | + | + |
| 31B-1 | − | − | + | + | +++ | +++ | +++ | +++ | +++ | +++ |
| 32B-3 | − | − | ++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ |
| 32B-4 | − | − | + | + | ++ | ++ | ++ | ++ | ++ | ++ |
| 32B-6 | − | − | + | + | ++ | ++ | ++ | ++ | ++ | ++ |
| 32C-3 | − | − | + | + | + | ++ | ++ | + | + | ++ |
| 32D-4 | − | − | + | + | +++ | +++ | +++ | +++ | +++ | +++ |

[a] Agglutination is recorded on a scale of − to +++.
[b] Macaque represents Japanese monkey and crab-eating monkey.

TABLE 2—*ELISA for identification of human blood based on the inhibition of reaction of hybridoma antihuman IgG with human IgG on the solid phase.*[a]

| Control (PBS) | Man | Chimp | Japanese Monkey | Crab-Eating Monkey | Baboon | Dog | Bull | Hog | Rat | Rabbit | Snake |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.61 | 0.14 | 0.40 | 1.40 | 1.37 | 1.43 | 1.54 | 1.53 | 1.54 | 1.09 | 1.41 | 1.43 |

[a] Values indicate absorbances at 490 nm.

[2] Y. Tamaki, N. Takahashi, T. Kishida, and K. Ishikawa, "Production and Characterization of Monoclonal Anti-G3m(21) Antibody," *Japanese Journal of Legal Medicine*, (submitted for publication).

For the immunologic identification of human blood, the specificity of the reagent being used is an essential requirement. Since some proteins of man and various animal species share antigenic determinants, heteroantisera to such human proteins usually exhibit cross-reactivity, particularly with the blood of nonhuman primates. The cross-reactive antisera are provided with the desired antibody specificity in three ways. Firstly, unwanted antibodies are absorbed with the blood of animals particularly primates, but it is not always available. Secondly, suitable antisera can be raised in primates [*3,4*]. Again, not all forensic science laboratories can afford to use the animals. Thirdly, high dilutions of unabsorbed antisera can be used in latex agglutination-inhibition [*5*] or ELISA [*6*].

We have presented a fourth method here. The introduction of the hybridoma technique by Köhler and Milstein in 1975 [*7*] opened up a new era in the history of immunology. The technology has since been diversified, and monoclonal antibodies have been used widely in immunologic studies. To our knowledge, however, no report has been made of their application to forensic science practice. We have been making an effort to establish hybridoma clones producing anti-Gm antibodies. During the growth of hybrid cells, we incidentally obtained an antihuman IgG antibody that was suitable for the identification of human blood. The generation and maintenance of monoclonal antibody-producing cell lines is so time-consuming that it is not advisable to use the hybridoma technique for species identification alone. Monoclonal antibodies, however, have the advantage of high titer, monospecificity, and reproducibility. Hence, they are a valuable tool for identification and quantitation of various antigens and haptens with which the forensic scientist must deal. It is hoped that many hybrid cell lines will be established for use in forensic science studies as well.

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