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G3m(21) Typing by ELISA and Dot Immunobinding with Enzyme-Labeled Monoclonal Anti-G3m(21) Antibody*

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Summary. Simple, rapid methods are described for G3m(21) typing with peroxidase-labeled monoclonal anti-G3m(21) antibody. In G3m(21) typing by ELISA, microtiter wells were coated directly with the test antigen, which was detected with the enzyme-labeled monoclonal antibody. To further simplify the procedure, a dot immunobinding method was developed. The antigen in the test serum applied onto a nitrocellulose membrane was successfully detected with the enzyme-labeled monoclonal antibody. These methods, particularly the dot immunobinding, are suitable for forensic casework because they are rapid and simple and require no technical skill.

Key words: Forensic immunology, Gm – Gm typing, ELISA, Dot immunobinding – Monoclonal antibody

Zusammenfassung. Es werden einfache schnelle Methoden zur G3m(21)-Typisierung mit enzymisch markierten monoklonalen Anti-G3m(21)-Antikörper beschrieben. Bei G3m(21)-Typisierung mit ELISA wurden Mikrotitertüpfel mit zu untersuchendem Antigen direkt überzogen. Das Antigen wurde mit dem enzymisch markierten monoklonalen Antikörper nachgewiesen. Eine Punkt-Immunobindung Methode wurde geschaffen, um das Prozedere noch weiter vereinfachen zu können. Das Antigen in der Probe wurde auf einer Nitrozellulosemembran appliziert und mit dem enzymisch markierten monoklonalen Antikörper erfolgreich nachgewiesen. Diese Methoden, besonders die Punkt-Immunobindung, sind für die forensische Praktik geeignet.

Schlüsselwörter: Forensische Immunologie, Gm – Gm-Typisierung, ELISA, Punkt-Immunobindung – Monoklonale Antikörper

Introduction

Since Grubb's discovery of G1m(1) [4], a considerable amount of information on the Gm system has been gained. It is now established that the Gm system is

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second only to the HLA system in genetic complexity and therefore in usefulness in forensic casework. Nevertheless, in some countries including Japan, only a limited number of forensic institutions have been making routine use of the Gm system. This is due chiefly to a shortage of Gm typing reagents, but partly to the conventional indirect hemagglutination-inhibition method being somewhat roundabout.

At present seven kinds of anti-Gm sera are commercially available (Biotest, Frankfurt/Main, FRG) in Japan. These antisera, i.e., anti-G1m(1), anti-G1m(2), anti-G1m(3), anti-G3m(5), anti-G3m(11), anti-G3m(14), and anti-G3m(21), can classify the Gm phenotypes of the Japanese into eight groups. However, neither anti-G3m(16) nor the corresponding anti-Rh_0 coat, to detect the G3m(15,16) antigen with, is generally available, although this marker is characteristic of the Mongoloids and is possessed by as many as 46.7% of the Japanese.

To solve these problems, we prepared rabbit anti-Gm sera, which enabled us to distinguish the nine Gm phenotypes in the Japanese [6, 9]. Subsequently, we developed an inhibition ELISA to effectively use the prepared antisera without the help of anti-Rh_0 coats [7]. Thus, we could type for G1m(3) and G3m(16) with the rabbit anti-Gm sera, and for G3m(21) with the monoclonal anit-G3m(21) antibody produced in our laboratory. With the world-wide popularity of ELISA methods in research and clinical laboratories, the forensic application of ELISA has been widened and diversified in recent years. Within the scope of Gm typing alone, other workers also have described ELISA methods. Fletcher et al. [1] and Francois-Gerard and Hoste [2] independently devised ELISAs for G1m(3) typing of sera and bloodstains with monoclonal anti-G1m(3) antibody. They obtained satisfactory results by a double-antibody method and a double-sandwich method, respectively.

Our inhibition ELISA involved the coating of wells with purified IgG of known Gm phenotype. To simplify the operation of ELISA, we have recently coated wells directly with the test antigen. Alternatively, to further simplify the method of Gm typing, we have developed a dot immunobinding (DIB) method. While derived from the now popular immunoblotting technique, our DIB method is an improved version of the ELISA involving direct immobilization of the test antigen. This method never became feasible until a monoclonal anti-G3m(21) antibody was produced. Here we describe primarily the procedure of DIB and compare it with the ELISA involving direct antigen immobilization in the hope that the DIB will be a model for the simplest possible method of Gm typing.

Materials and Methods

Monoclonal Antibody

Monoclonal anti-G3m(21) antibody 200D1 (MCAb21) was produced by the hybridoma technique [3], as described previously [10]. The MCAb21 in ascitic fluid had a titer of 1:500,000 against a 0.2% suspension of anti-Rh_0-coated cells. The MCAb21 was found to be of IgG1 in the Ouchterlony test with anti-subclass sera (Zymed Laboratories, San Francisco, CA, USA). The Ig fraction of antibody-containing ascitic fluid was obtained by ammonium sulfate precipita-

tion. Part of the Ig fraction was labeled with horseradish peroxidase (PO) by the periodate method [8].

Test Sera

Serum samples were taken from healthy individuals living in the western part of Japan and Gm-typed by the conventional hemagglutination-inhibition method. To remove albumin and other serum proteins, test sera diluted ten times with 0.01 *M* phosphate buffer, pH 7.6, were absorbed for 15 min with an equal volume of DE52 (Whatman, Kent, UK) equilibrated with the same buffer. The supernatant contained only IgG as detected by the Ouchterlony method.

ELISA

Eighty G3m(21)-positive serum samples and 20 G3m(21)-negative serum samples were subjected to an ELISA. The whole procedure was carried out at room temperature. Aliquots (100 µl) of the absorbed test sera were pipetted into the wells of a polystyrene microtiter plate (Sumitomo Bakelite, Tokyo, Japan). After incubation for 1 h, the wells were washed three times with 0.01 *M* phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween), pH 7.4. To each well was added 80 µl of an appropriate dilution of the PO-MCAb21 in PBS-Tween. After incubation for 1 h followed by three washes, 80 µl of a substrate mixture was added to each well. The substrate mixture was prepared by adding 50 mg of *o*-phenylenediamine dihydrochloride and 20 µl of 30% hydrogen peroxide to 100 ml of 0.05 *M* phosphate-0.024 *M* citric acid buffer, pH 5.0. The plate was left in the dark for 15 min, and the enzymic reaction was stopped by the addition of 80 µl of 12.5% sulfuric acid. The absorbance of the colored product was measured at 490 nm on a MICROELISA Minireader MR590 (Dynatech, Alexandria, VA, USA).

Alternatively, the wells were coated with unabsorbed, PBS-diluted test sera, and reacted with an appropriate dilution of the PO-MCAb21.

Dot Immunobinding

Both raw and DE52-treated sera from a total of 100 subjects were used: 80 were G3m(21)-positive and 20 were G3m(21)-negative. All the steps were carried out at room temperature. Aliquots (0.5 µl) of samples were applied onto a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA, USA). The membrane was air-dried and reacted with an appropriate dilution of the PO-MCAb21 in 0.02 *M* Tris-0.5 *M* saline containing 0.5% bovine serum albumin and 0.05% Tween 20 (TBS-Tween), pH 7.5, for 30–60 min. The membrane was washed twice with TBS-Tween. After a brief wash with distilled water, the membrane was immersed in a color-developing solution for 15 min. The solution was prepared by mixing 30 mg 4-chloro-1-naphthol, 10 ml methanol, 50 ml TBS, and 25 µl of 30% hydrogen peroxide. The enzymic reaction was stopped by washing the membrane with tap water.

Results and Discussion

Figure 1 shows the results of a typical ELISA for serum samples absorbed with DE52. Each dot represents the absorbance value for a single sample. The mean value for the G3m(21)-positive samples was 2.21 with a range of 1.40 to 2.76, while the mean value for the G3m(21)-negative samples was 0.43 with a range of 0.21 to 0.76. The positive and negative samples could be readily distinguished from each other with the naked eye. Since there are individual variations, it is preferable to assay each sample in duplicate or triplicate.

When wells were coated with raw sera, absorbance values varied so widely that positives could not be distinguished from negatives. Thus, in this ELISA system, one cannot omit absorption of test sera with DE52.

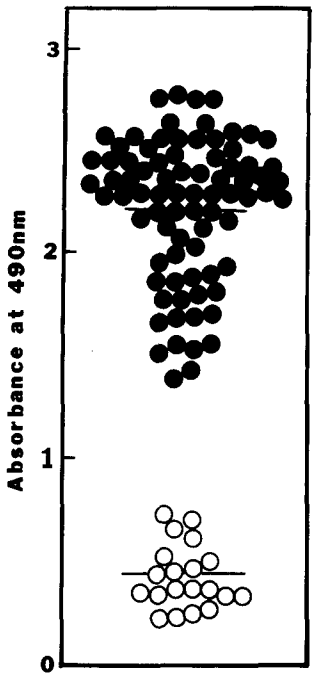

Fig. 1. Gm typing of DE52-absorbed sera by ELISA. The mean value for the G3m(21)-positive samples (●) is 2.21 (range 1.40 to 2.76), the mean value for the G3m(21)-negative samples (○) is 0.43 (range 0.21 to 0.76). Each *dot* represents the absorbance for a single sample. *Horizontal bars* indicate mean values

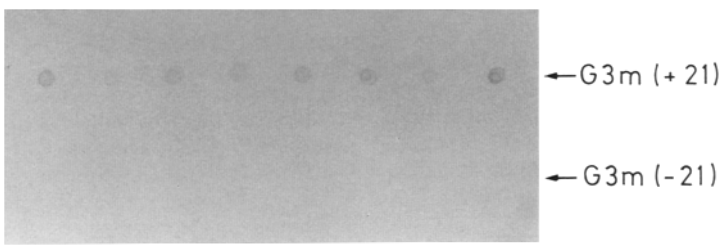

Fig. 2. Gm typing of DE52-absorbed sera by dot immunobinding

Figure 2 depicts the DIB results for the serum samples absorbed with DE52. The G3m(21)-positives could be clearly distinguished from the G3m(21)-negatives by this method.

When samples of undiluted whole serum were applied to a nitrocellulose membrane, the positive dots were pale-colored to the extent that it was frequently difficult to discriminate between positives and negatives. This was probably because other serum proteins, particularly albumin, prevented adsorption of IgG3 which occupies only 8% of the IgG in normal serum. In contrast, when serial double dilutions of serum (1:2 to 1:512) were applied, dilutions of 1:4 to 1:32 gave the most satisfactory results although positives could be distinguished from negatives with the other dilutions tested.

In the previously described inhibition ELISA [7], we had to prepare purified IgG of different Gm phenotypes to precoat microplate wells with. In the present study to simplify the procedure, we coated wells with DE-absorbed (i.e.,

the IgG fraction of) test sera, and could successfully detect the G3m(21) antigen with PO-MCAb21.

To further simplify the procedure, we directly immobilized whole serum in wells, but ended up with failure as mentioned earlier. Therefore, we switched to a DIB method, and with success.

The most common problems with ELISA using polyclonal antibody are non-specific adsorption and cross-reaction of the antibody, both leading to a high background signal. In determination of isotypic antigens, both problems can easily be solved by use of affinity-purified antibodies. In contrast in allotyping, such as Gm typing, the problems are not easy to solve. While cross reaction can be kept within permissible limits by adoption of an inhibition ELISA [7], non specific adsorption in ELISA involving direct antigen immobilization can not, in our experience, be removed even by use of affinity-purified antibody. It can be solved only by using monoclonal anti-allotype antibody.

Needless to say, the use of monoclonal antibody is essential to the DIB method for Gm typing. It has several advantages: (a) the test serum can be directly applied even if it contains an anti-IgG antibody that interferes with Gm typing by the conventional technique, (b) the procedure is very simple and requires no technical skill, (c) the entire procedure can be carried out within 1 h if the enzyme-labeled monoclonal antibody is available, (d) the test results can be preserved semipermanently, and (e) it is a potentially versatile method for allotyping of any serum proteins if monoclonal antibodies become available. For this reason, the method is suited for Gm typing particularly in forensic casework. We hope that the DIB will be a model for the simplest possible method of Gm typing. To our knowledge, however, only a few Gm monoclonals have been produced [5; Jefferis, personal communication]. We will have to wait for some time for several other Gm monoclonals to be produced before the DIB becomes a routine method of Gm typing.

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