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Prevalence of human anti-mouse antibodies (HAMAs) in routine examinations

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ABSTRACT

Background: Circulating heterophilic antibodies interfere with immunological assays in laboratory examinations; however, their rate of incidence is currently questionable. We developed an enzyme-linked immunosorbent assay (ELISA) to detect human anti-mouse antibodies (HAMAs) in routine examinations.
Methods: The study samples were comprised of serum samples obtained from 290 inpatients and outpatients at our hospital. Mouse immunoglobulin G1 (mIgG1), mIgG2a, and mIgG2b were used as the antigens and horseradish peroxidase (HRP)-conjugated anti-human IgG and IgM were used to identify the HAMA isotype.
Results: HAMAs were detected in 11.7% (34/290) of the samples. We observed 18 and 20 samples positive for IgG- and IgM-type HAMAs, respectively. Four samples contained both IgG- and IgM-type HAMAs. HAMAs against mIgG1, mIgG2a, and mIgG2b were found in 21, 14, and 13 samples, respectively. Existence of HAMAs was confirmed by western blotting using mIgG's as the antigens and HAMAs as the primary antibodies. Heterophilic blocking reagent (HBR) was also used to block the heterophilic interactions. Unexpectedly, a low HBR concentration rather enhanced the interactions instead of blocking them.
Conclusions: A considerable number of HAMA-positive samples, reacting with the heavy chain of mIg, were found in routine examinations. A sufficient amount of HBR should be used for blocking the heterophilic interactions.

1. Introduction

Unexpectedly, human serum contains anti-mouse, anti-rabbit, anti-sheep, anti-goat, anti-bovine, or anti-swine antibodies. These circulating heterophilic antibodies in the human serum interfere with immunological assays, including thyroid-stimulating hormone (TSH), a-fetoproteins (AFP), cancer antigen 125 (CA-125), creatine kinase-muscle and brain (MB) isozyme, and elastase 1 assays [1–5]. It falsely enhances or mitigates the responses by bridging or blocking the antibodies in the reagents, respectively; this leads to misdiagnosis and unnecessary treatments. Reportedly, the presence of heterophilic antibodies is etiologically associated with blood transfusion, vaccination, administration of therapeutic animal antibodies, certain disease conditions, and other unknown mechanisms [6]. The prevalence of anti-animal antibodies in normal subjects is ambiguous; it is widely estimated to be between 1% and 80% [6]. One of the reasons is that most of the studies reporting the prevalence of heterophilic antibodies were conducted in the last century, and the existence of the antibodies was determined on the basis of the difference in the values obtained for the samples with or without absorption of the heterophilic antibodies by certain animal antibodies [1,2,4]. Thus, an appropriate detection system is required to detect anti-animal antibodies as well as gather information regarding the characteristics and prevalence of heterophilic antibodies.

2. Patients and methods

2.1. Serum samples

In 2007, 290 serum samples were randomly collected from inpatients and outpatients at our hospital after obtaining the written informed consent of all the patients. There were 161 male and 129 female patients and the age was between 8 and 90 years (mean = 61.0 years). None of the patients received therapeutic or diagnostic monoclonal antibody before this study.

2.2. Selection of antibodies for coating

Mouse-IgG1 (mIgG1; X0931) and mIgG2a (X0943) were from DAKO (Japan, Tokyo). mIgG1 (M9269, M9035), mIgG2a (M5409) and mIgG2b (M5534) were purchased from Sigma (Japan, Tokyo). The murine immunoglobulins (5 mg/ml; total volume, 100 ml) were coated in a 96-well enzyme immunoassay (EIA)/radioimmunoassay (RIA) plate (Corning, NY) and left overnight at 4 °C. They were then removed, and

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the wells were blocked with Block Ace (DS Pharma Biomedical, Japan, Osaka) for overnight at 4 °C. After removing the blocking reagent, the wells were washed 3 times with phosphate-buffered saline supplemented with 0.02% Tween-20 (PBS-T). They were then incubated with 100 ml of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody (1.3 mg/ml Dako; P0260) for 30 min at room temperature (RT). After incubation, the wells were washed 4 times with PBS-T, incubated with orthophenylenediamine (DAKO) for 20 min, and the reaction was terminated with 1 mol/l sulfuric acid. The absorbance of 490 nm was measured using an Infinite 2000 plate reader (TECAN, Japan, Kawasaki). From the results of the experiments conducted to identify the best condition showing the highest contrast between the negative controls and immobilized murine immunoglobulins, mIgG1 (M9035), mIgG2a (M5409) and mIgG2b (M5534) were chosen as the immunoglobulins to be used in further assays. All these 3 immunoglobulins contain k-type light chains.

2.3. Detection of HAMAs in patient serum samples

Patient serum was diluted to 1:100 in PBS and added to an ELISA plate in duplicates, constructed as described above, and incubated for overnight at 4 °C. The samples were then removed; the wells were washed 4 times with PBS-T and then incubated with 100 ml of either HRP-conjugated rabbit anti-human IgG antibody (DAKO; P0214) or IgM antibody (DAKO; P0215) for 30 min at RT. Subsequently, the wells were washed and incubated with the substrate, after which the reaction was terminated. The absorbance was measured as described above. The well without any coated antibody was used as the negative control, while that with the HRP-conjugated anti-mouse IgG antibody was used as the positive control. To adjust inter-test imprecision, the ratio to the positive control was calculated and used in this study. Cut-off values were determined using the mean + 2 SD. The cut-off values for IgG-type HAMA were 0.423, 1.165 and 0.690 for mIgG1, mIgG2a, and mIgG2b, respectively, while those for IgM-type HAMA were 0.301, 0.614 and 0.306 for mIgG1, mIgG2a and mIgG2b, respectively.

2.4. Confirmation of existence of HAMAs in patient serum samples

The existence of HAMAs was confirmed by western blotting. In brief, 1.5 mg of mIgG1, mIgG2a, and mIgG2b were electrophoresed in 4%–20% gradient Tris-glycine gel (Invitrogen, Japan, Tokyo), transblotted to nitrocellulose membranes, and blocked with Block Ace for 1 h at RT. The patient serum was diluted to 1:100 in Block Ace and incubated overnight at 4 °C. The membranes were washed and developed using a Western Breeze chemiluminescent western blot immunodetection kit (Invitrogen), according to the manufacturer's instructions. Images were obtained using the ChemiDoc XRS System (BioRad, Japan, Tokyo).

2.5. Blocking of heterophilic interactions in ELISA

To confirm that the positive results in ELISA are due to heterophilic interactions, murine Ig's (heterophilic blocking reagent; HBR, Scantibodies Laboratories, Japan, Tokorozawa) were added to the serum samples before the assay.

2.6. Statistical analysis

Statistical analysis of the data was carried out using Microsoft Excel®. Statistical significance (defined as $p<0.05$) was evaluated with Student's *t*-test.

Table 1
IgG-type HAMA in patient serums.

Sample No.	Coated antigens		
	mIgG1	mIgG2a	mIgG2b
126	0.526	2.687	1.451
138	0.336	2.679	1.635
172	0.045	4.625	3.020
252	0.067	5.859	3.253
61	0.684	0.061	0.042
73	0.441	0.210	0.144
141	0.461	0.082	0.044
143	0.484	0.164	0.119
236	0.565	0.067	0.109
64	0.807	0.048	0.030
77	0.587	0.201	0.133
96	0.427	0.090	0.048
112	0.503	0.332	0.161
115	0.662	0.314	0.248
128	0.483	0.016	0.015
142	0.483	0.564	0.468
213	0.426	0.081	0.017
97	0.071	1.236	0.560

Positive values are underlined. ($n=2$).

3. Results

3.1. Detection of HAMAs in patient serum samples

A total of 290 serum samples were tested for HAMAs by using an ELISA plate coated with mIgG1, mIgG2a, or mIgG2b. As shown in Table 1, 18 (6.2%) samples were found to contain IgG-type HAMA. Of these, 1 sample reacted with mIgG1, mIgG2a, and mIgG2b; 3 reacted with mIgG2a and mIgG2b; 13 reacted with mIgG1; and 1 reacted with mIgG2a. IgM-type HAMA was found in 20 (6.9%) samples. Of these, 2 samples reacted with mIgG1, mIgG2a and mIgG2b; 5 reacted with mIgG2a and mIgG2b; 9 reacted with mIgG1; 2 reacted with mIgG2a; and 2 reacted with mIgG2b (Table 2). Among these samples, sample 73, 141, 143, and 236 were detected to be positive for both IgG- and IgM-type HAMAs.

Overall, 34 (11.7%) samples were found to be positive for either IgG- or IgM-type HAMA. Of those positive for IgG-type HAMA, 21 (7.2%), 14 (4.8%), and 13 (4.5%) samples reacted with mIgG1, mIgG2a, and mIgG2b, respectively. The prevalence is summarized in Table 3.

Table 2
IgM-type HAMA in patient serums.

Sample No.	Coated antigens		
	mIgG1	mIgG2a	mIgG2b
36	0.421	0.886	0.773
168	0.345	1.338	0.624
122	0.228	1.101	0.541
131	0.143	0.684	0.308
173	0.062	1.633	0.538
220	0.235	1.394	0.611
270	0.109	1.472	0.827
73	0.328	0.104	0.052
141	0.387	0.119	0.139
143	0.353	0.036	0.017
236	0.304	0.247	0.082
19	0.488	0.233	0.095
39	0.584	0.190	0.108
51	0.823	0.604	0.133
74	0.339	0.100	0.061
137	0.380	0.002	0.026
285	0.113	0.892	0.300
289	0.103	0.641	0.235
94	0.084	0.452	0.398
174	0.051	0.126	0.341

Positive values are underlined. ($n=2$).