High Prevalence of Human Anti-mouse Antibodies in the Serum of Colorectal Cancer Patients

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Abstract. *Background: Monoclonal antibody treatment induces the expression of human anti-mouse antibodies (HAMA), which in turn interfere with the therapy. However, whether HAMAs are expressed before the initiation of antibody therapy in patients with colorectal cancer remains unknown. Materials and Methods: Serum samples were collected from 40 patients diagnosed with colorectal cancer. Serum samples from 157 individuals without cancer were used as controls. None of the patients received imaging or therapeutic antibodies before the study. The expression of HAMAs was evaluated by ELISA with murine immunoglobulin G1 (mIgG)1, mIgG2a and mIgG2b as the antigen. Results: Of the 40 colorectal cancer patients, 9 (22.5%) expressed either IgG- or IgM-type HAMAs while only 13/157 (8.2%) of the individuals without cancer expressed the HAMAs ($p<0.05$). Conclusion: HAMAs are prevalent in the serum of colorectal cancer patients even before antibody administration. Medical practitioners should be alert to the possibility of HAMA expression when administering antibody therapy.*

Human anti-mouse antibodies (HAMAs) are heterophil antibodies that are expressed in human serum and can interfere with immunoassays (1), including the measurement of cancer antigen 125 (CA-125) and carcinoembryonic antigen (CEA) (2, 3). HAMAs falsely enhance or mitigate the responses by bridging or blocking the antibodies in the reagents, respectively; this leads to misdiagnosis and unnecessary treatments. It also interferes with monoclonal antibody-based therapy by inducing adverse events such as infusion reactions and may neutralize the effects of the therapeutic antibodies (4, 5). The administration of murine monoclonal antibodies induces HAMA responses; the incidence of such responses is reported to be between 8-88% (6-10).

We recently reported that HAMAs are expressed in 11.7% of serum samples tested in routine examinations (11). In the previous study, we found that the incidence of HAMAs is higher in patients diagnosed with cancer than in those without cancer (15.8% *vs*. 8.3%, $p<0.05$); these patients were not treated with imaging or therapeutic antibodies before the study. In the present study, we examined the prevalence of HAMAs in patients with colorectal cancer who had not previously been administered murine immunoglobulins.

Materials and Methods

Serum samples. In 2009, serum samples were collected from 40 Japanese colorectal cancer patients who provided their written informed consent. The study involved 21 men and 19 women aged between 46 and 86 years (mean=68.8 years). The tumours were staged according to the system of American Joint Committee on Cancer. Serum samples from 157 individuals without cancer were collected for use as controls. None of the patients were treated with imaging or therapeutic antibodies before the study.

Detection of HAMAs in serum samples. HAMAs were detected using a previously described method (11). In brief, the serum samples were diluted in phosphate-buffered saline (PBS), added to an ELISA plate coated with murine immunoglobulin G (mIgG)1, mIgG2a, or mIgG2b as the antigen and incubated overnight at 4˚C. After incubation, the plates were washed 4 times with PBS

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Abbreviations: CA-125: cancer antigen 125; CEA: carcinoembryonic antigen; HAMA: human anti-mouse antibodies; HRP: horseradish peroxidase; mIg: murine immunoglobulin; PBS: phosphate-buffered saline; RT: room temperature.

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Table I. *Prevalence of HAMAs in the patients diagnosed with or without colorectal cancer.*

	Patients without cancer	Patients with colorectal cancer
Total	157	40
HAMA-positive	13 (8.2%)	9 (22.5%)
IgG-HAMA	5 (3.1%)	4 (10.0%)
IgM-HAMA	8 (5.1%)	5 (12.5%)

containing 0.02% Tween 20, incubated with 100 μl of either horseradish peroxidase (HRP)-conjugated anti-human IgG antibody or HRP-conjugated anti-human IgM antibody (DAKO, Japan, Tokyo) for 30 min at room temperature (RT), and then incubated with orthophenylenediamine (DAKO) for 20 min at RT, after which the reaction was terminated with 1 M sulfuric acid. The absorbance at 490 nm was measured using an Infinite 2000 plate reader (TECAN, Japan, Kawasaki). Wells that were not coated with any immunoglobulin were used as negative controls, whereas those treated with HRP-conjugated rabbit anti-mouse IgG antibody were used to create reference values. Cut-off values were determined using the mean plus 2 standard deviations of the samples in routine examination, as described previously (11). The cut-off values of IgG-type HAMAs against mIgG1, mIgG2a, and mIgG2b were 231 μg/l, 907 μg/l, and 701 μg/l, respectively. The cut-off values of IgM-type HAMAs against mIgG1, mIgG2a, and mIgG2b were 176 μg/l, 489 μg/l, and 317 μg/l, respectively.

Blocking experiments using Fab and Fc. To identify the immunoglobulin fragments responsible for the reaction with HAMAs, mIg $F(ab')_2$ and Fc fragments were added to the ELISA assay. The $F(ab')_2$ and Fc fragment of mIg purified from mouse sera were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Preliminary experiments revealed that 10 μg of the Fc or $F(ab')_2$ fragment was sufficient to block the reaction of HRP-conjugated rabbit anti-mouse IgG.

Statistical analysis. The data were statistically analyzed using Microsoft Excel®. Statistical significance (defined as $p<0.05$) was evaluated using Pearson's chi-square test.

Results

High prevalence of HAMAs in the sera of colorectal cancer patients. Of 40 colorectal cancer patients, 9 (22.5%) expressed either IgG- or IgM-type HAMAs, which was significantly higher than in those without cancer (8.2%, $p<0.05$; Table I). The mean age of the patients with HAMAs was 68.5 years. No HAMAs were found in the sera of the patient with stage I cancer (Table II). Female patients showed higher prevalence of HAMAs than male patients (3/21 *vs*. 6/19); however, the difference was not statistically significant. Expressions of HAMAs were observed in the sera of patients with tubular, and mucinous adenocarcinoma (Table III).

Table II. *Stages of colorectal cancer and the prevalence of HAMAs.*

	HAMA-positive
I	0/5
II	4/16
III	3/10
IV	2/9

Table III. *Histology of colorectal cancer and the prevalence of HAMAs.*

	HAMA-positive
Tubular adenocarcinoma	7/30
Poorly differentiated adenocarcinoma	0/4
Mucinous adenocarcinoma	2/5
Endocrine cell cancer	0/1

Heterogeneity of HAMAs in the sera of colorectal cancer patients. The types of HAMAs detected in the sera of the colorectal cancer patients were characterized. As shown in Table IV, four IgG-type HAMAs were identified. Of these, three only recognized mIgG1 and one reacted with all forms of mIgG tested. Five IgM-type HAMAs were identified. Of these, three recognized mIgG1, one recognized mIgG2a, and one recognized all forms of mIgG tested. Overall, HAMAs against mIgG1 were most prevalent and those against mIgG2b least prevalent. This tendency was also seen in HAMAs in the sera of the control individuals that HAMAs against mIgG1 were most prevalent and those against mIgG2b least prevalent (data not shown).

HAMAs recognizing various mIg epitopes. To identify the immunoglobulin fragments recognized by the HAMAs, murine immunoglobulin fragments $F(ab')_2$ and Fc were used to block the HAMA reactions. Of six IgG-type HAMA reactions, five and two reactions were blocked by the F(ab')2 and Fc fragment, respectively (Table V). In sample no. 2, $F(ab')_2$ and Fc fragments were able to block the HAMA reaction. However, both fragments were required for blocking the reaction, completely. Of seven IgM-type HAMA reactions, six were blocked by the $F(ab')_2$ and Fc fragments, respectively (Table V). $F(ab')_2$ and Fc fragments alone were able to block most of the HAMA reactions; however, both fragments were required to block the reaction completely. These results indicate that HAMA reactions are heterogeneous in the samples of patients who have not been administered therapeutic or imaging monoclonal antibodies.

Table IV. *HAMAs present in the sera of colorectal cancer patients.*

IgG-type HAMAs.

	Coating antigen (μg/l)		
Patient no.	mIgG1	mIgG2a	mIgG2b
1	**241**	312	380
2	**429**	594	340
3	**248**	96	74
4	**325**	**1002**	**880**

IgM-type HAMAs

	Coating antigen (μg/l)		
Patient no.	mIgG1	mIgG2a	mIgG2b
5	**201**	261	124
6	**218**	96	135
7	**248**	238	123
8	**335**	**515**	**437**
9	83	**609**	173

Positive results above cut-off levels are given in bold.

Table V. Recognition sites of HAMAs in the sera of colorectal cancer patients.

IgG-type

Patient no.	IgG subclass	$F(ab')_2$	Fc
1	IgG1	+	–
2	IgG1	+	+
3	IgG1	–	+
4	IgG1	+	–
	IgG2a	+	–
	IgG2b	+	–

IgM-type

Patient no.	IgG subclass	$F(ab')_2$	Fc
5	IgG1	+	+
6	IgG1	+	+
7	IgG1	+	–
8	IgG1	+	+
	IgG2a	+	+
	IgG2b	+	+
9	IgG2b	–	+

+, Blocked by the peptide; –, not blocked by the peptide.

Discussion

In this study, we found that 22.5% of the patients diagnosed with colorectal cancer expressed HAMAs in their serum at significantly higher prevalence than those expressed in individuals without cancer. We previously reported that the incidence of HAMA is higher in patients diagnosed with cancer than in those without cancer (11). However, the type of cancer that is associated with HAMA has not been identified. We chose patients with colorectal cancer for this study as these patients are occasionally treated with therapeutic monoclonal antibodies, including cetuximab and bevacizumab. To the best of our knowledge, this is the first study to evaluate the incidence of HAMAs in colorectal cancer patients who have not previously been treated with monoclonal antibodies. Our results indicate that adverse events associated with HAMAs may occur on initiation of antibody therapy.

Monoclonal antibody-based therapy has evolved as the conventional treatment of choice for many types of cancer (12, 13); the relevant drugs include bevacizumab, cetuximab, gemtuzumab, rituximab, and trastuzumab. All these antibodies are genetically engineered and rendered completely humanized or chimeric in order to reduce immunogenicity to avoid a HAMA response.

In this study, we found that the HAMA subtypes as well as the mIg epitopes recognized by the HAMAs were heterogeneous. mIgG1 was the most immunogenic and mIgG2b the least immunogenic mIg recognized by the HAMAs expressed in 9 serum samples. Of 13 HAMA reactions, 11 were blocked by the $F(ab')_2$ fragment of mIgG and 8, by the Fc fragment. These data might be useful for the bioengineering of therapeutic antibodies.

There is controversy whether the expression of HAMAs has benefits or disadvantages for survival of the patients (7). Several reports show that the induction of HAMA expression after the administration of therapeutic antibodies improves the patient's prognosis (6, 10, 14). A possible explanation for this is that therapeutic monoclonal antibodies evoke host immune responses, including antibody-dependent cellular cytotoxicity, complement activation, induction of adaptive immune responses, and stimulation of the idiotypic network to elicit tumor antigen-specific immune responses (15). In immunoassays, HAMAs enhance or mitigate the results by cross-linking or blocking antibodies in the reagents; this activity depends on the nature of the HAMAs and the reagents used. Not only the HAMAs existing before antibody administration but also the HAMA responses after administration of therapeutic antibodies are heterogeneous (9, 14); we speculate that the nature of the interactions of HAMAs and therapeutic immunoglobulins will determine whether HAMAs enhance or hamper antibody therapy.

The mechanism underlying the expression of HAMAs in colorectal cancer patients remains elusive. Factors other than monoclonal antibody treatment, such as blood transfusions (16), vaccinations (17), animal husbandry (18), and

multiparity (16), are known to trigger the expression of heterophilic antibodies; however, none of these factors were applicable to the patients in this study. Neoplasms are considered to be associated with various syndromes caused by auto-antibodies, that is, paraneoplastic syndromes (19). Similar mechanisms may govern the expression of HAMAs in colorectal cancer patients.

In conclusion, HAMAs are prevalent in the serum of colorectal cancer patients, even before monoclonal antibody administration. Staff in clinical laboratories should be aware of the existence of HAMAs when encountering unexpected results using the serum of colorectal cancer patients in immunoassays. Medical practitioners should also be alert to the possibility of HAMA expression when administering antibody therapy and consequent adverse reations.

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