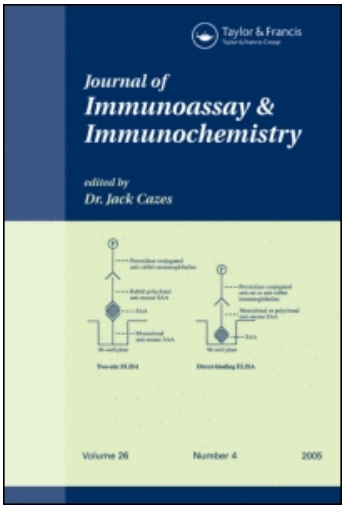

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Non-Specific Immunoglobulin Interactions May Lead to False-Positive Results in Assays for Human Anti-Mouse Monoclonal Antibodies (HAMA)

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NON-SPECIFIC IMMUNOGLOBULIN INTERACTIONS MAY LEAD TO FALSE-POSITIVE RESULTS IN ASSAYS FOR HUMAN ANTI-MOUSE MONOCLONAL ANTIBODIES (HAMA)

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ABSTRACT

This abstract demonstrates that non-specific immunoglobulin interactions, of the $F(ab')_2$ region and Fc piece of human IgG may lead to false-positive results in patients being monitored for naturally occurring (pre-existing) or induced human anti-mouse monoclonal Ig antibodies (HAMA). Patient sera tested before administration of murine monoclonal antibodies for immunoscintography or immunotherapy showed 'positive' results when screened by specific ELISA for the presence of pre-existing antibodies to murine immunoglobulin. However, only serum from a patient which had previously received murine monoclonal antibody for immunotherapy, was it possible to show specificity by competitive inhibition. Thus, the use of competitive inhibition by specific antigen (mouse Ig isotype/subtype), sera positive for HAMA or pre-existing HAMA can be distinguished from false-positive reactions. (KEY WORDS: Cancer, ELISA, HAMA, heterophilic, monoclonal, immunotherapy).

INTRODUCTION

During the last decade, the use of murine monoclonal antibodies (mmAb) for cancer immunotherapy, localization and *in vivo* diagnosis has begun to be widely used (1). As more patients are treated with mmAb, the incidence of human anti-mouse monoclonal antibodies (HAMA) will increase as well. It is important to monitor the immune response of patients against the administered mmAb, especially if more than one-round of therapy is planned, because the effectiveness of subsequent cycles of therapy may be compromised by circulating antibodies against the mouse monoclonal antibody (2,3).

Therefore, it should become standard practice that before patients are given a second cycle of mmAb, they be tested for the presence of circulating HAMA.

The occurrence in humans, of pre-existing antibodies to a variety of animal proteins has been shown (4), especially to mouse IgG (5). For this reason many patients are tested before any mmAb is administered for localization of tumors, diagnosis, or immunotherapy. A positive test for the presence of pre-existing or induced HAMA may well influence the course and type of cancer treatment employed. This report describes studies which show that non-specific protein-protein interactions, particularly through the Fc piece and $F(ab')_2$ region of human IgG may appear to be 'specific' for HAMA, but may not in fact be specific, resulting in false positive results possibly leading to patients being deprived of potentially beneficial immunotherapy. In addition, such protein-protein interactions through the Fc piece of IgG may also mislead to interpretations for the presence of heterophilic antibodies (6).

MATERIALS AND METHODS

Microtiter plates (96-well, Falcon) were coated with 50 μl/well mouse-IgG (5 mg/ml phosphate-buffered saline). The plates were dried overnight at 37°C. The wells were then blocked with 10% fetal calf serum in phosphate-buffered saline (PBS) and incubated for 1½ hours at room-temperature (RT) or overnight at 4°C. The plates were then washed 4 times with 0.05% Tween-20/PBS. Next an equal volume of serum or IgG fraction of serum was added to various concentrations of inhibitor (mouse or porcine-IgG, [Sigma]) and incubated for 2 hours at RT. Fifty microliter of this inhibitor mixture was then added to the mouse-IgG coated wells and incubated for 1½ hours at RT. In the experiments which tested non-specific immunoglobulin interactions, varying concentrations of Fc or $F(ab')_2$ preparations

(Cappel) were added to the microtiter wells instead of serum samples. After washing the plates, 1/150 dilution of alkaline phosphatase conjugated goat anti-human IgG (Sigma) at 50 μl/well was added to the wells and incubated for 30 minutes at RT. The plates were then read at 405 nm (Titertek, FLOW).

RESULTS

During routine screening for pre-existing HAMA activity in serum from brain tumor patients who were candidates for mmAb mediated therapy or localization studies, a great deal of variation was noted among the serum from patients as to the levels of presumed HAMA activity. In **Figure 1**, four different serum samples are shown. Two samples, JC and LH, were from patients before exposure to mmAb and 2 samples from patient PS before (PS-pre-) and after (PS-post-) therapy with mmAb. Serum samples from JC and LH and pre- and post-therapy PS show varying degrees of binding to mouse-IgG coated plates. However, only the post-therapy sample from PS was able to be competitively inhibited with mouse-IgG. For all other samples, it was impossible to show any significant inhibition. Thus only with the post-therapy PS sample was it possible to demonstrate specific binding to mouse-IgG coated plates. These experiments were repeated with serum samples from other patients and healthy volunteers, with similar results (data not shown), confirming the findings shown in Figure 1, that it was not possible to inhibit the binding of the serum samples with mouse IgG.

In order to reduce the possible influence of non-IgG serum proteins which may contribute to the non-specific binding of serum to mouse IgG coated plates, IgG fractions of post-therapy PS serum and RP serum (healthy volunteer) were made by protein A column chromatography. **Figure 2** shows the results obtained with the IgG fractions tested on mouse-IgG coated plates. As in the previous

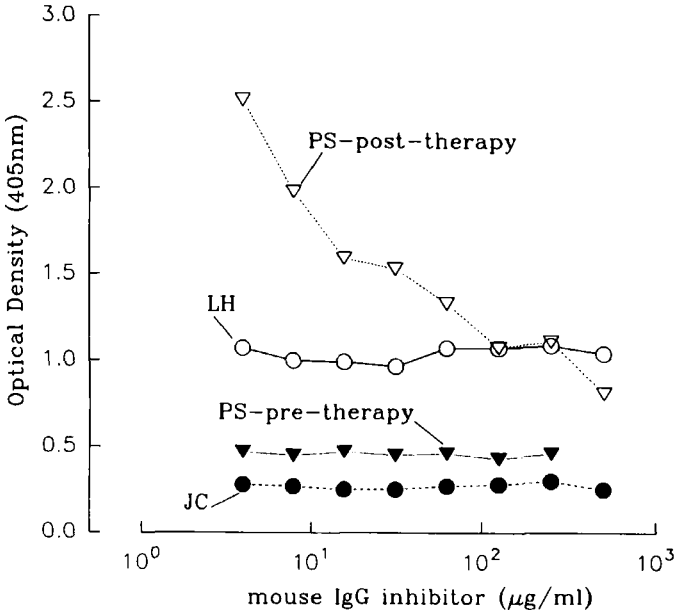

Figure 1. ELISA for HAMA. Microtiter wells coated with mouse IgG. Serum from PS-post-therapy (▽) can be specifically inhibited with mouse IgG, but not sera from LH (o), JC (●) and PS-pre-therapy (▾).

results (Figure 1), only the sample from post-therapy PS showed specificity, that is, binding being specifically inhibited with mouse IgG. The specificity for mouse IgG by the sample post-therapy PS was confirmed by the inability of porcine IgG to cause any significant inhibition.

To test whether human IgG could possibly account for the non-specific binding to mouse-IgG seen in patients and healthy volunteers, preparations of human IgG Fc and $F(ab')_2$ were tested for their binding to mouse-IgG coated plates. As can be seen in **Figure 3**, increasing concentrations of either Fc or $F(ab')_2$ preparations of

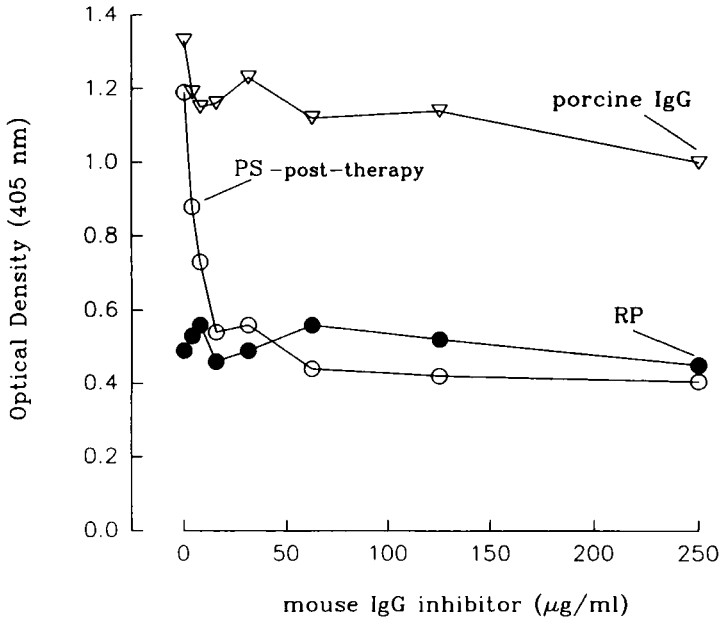

Figure 2. ELISA for HAMA. Microtiter wells coated with mouse IgG. Specificity of HAMA of PS-post-therapy serum (o) shown by ability of mouse IgG but not porcine IgG (▽) to competitively inhibit HAMA ELISA. RP (●):binding of serum from healthy volunteer which can not be inhibited with mouse IgG.

human IgG resulted in higher amounts being bound to the mouse IgG coated wells. This demonstrates that human IgG is capable of binding through the Fc region to mouse IgG in a non-specific manner, that is, not through the antigen-binding site.

DISCUSSION

The results presented here, especially those shown in Figures 2 and 3, strongly imply that human IgG, through the Fc piece and $F(ab')_2$ region, can interact non-specifically to mouse IgG coated wells, during an ELISA for determining HAMA. These non-specific

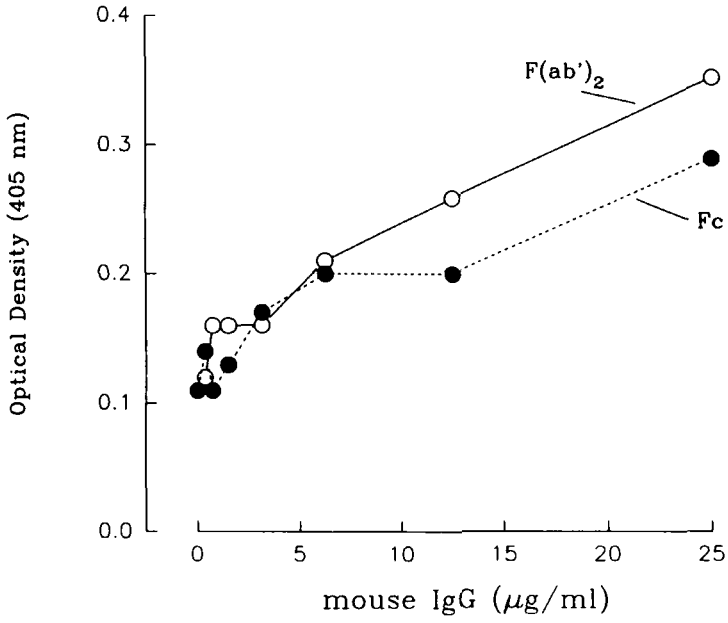

Figure 3. ELISA for HAMA. Preparations of $F(ab')_2$ (o) and Fc piece (●) bind non-specifically to microtiter wells coated with mouse IgG.

interactions may be misinterpreted for the presence of heterophilic antibodies. These results do not demonstrate that pre-existing antibodies to mouse Ig do not occur in individuals, as this has been convincingly demonstrated (4) however, it must be explicitly shown that the interaction of human serum samples and mouse Ig is specific for the antigen in question and that heterophilic antibodies are shown to bind their antigens through the antigen-binding site. Additionally, the presence of pre-existing antibodies to mouse Ig may not be detrimental to the course of therapy for the patient (7). Therefore, precautions must be taken in any ELISA or immunoassay which is designed to detect human antibodies to proteins such as

mmAb (IgG, IgM, ect), cytokines, peptide hormones, etc., to show specificity of the reaction being measured, to ensure that positive findings are distinguished from false positive and false negative results (5).

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