Circulating Antibodies to Mouse Monoclonal Immunoglobulins in Normal Subjects—Incidence, Species Specificity, and Effects on a Two-Site Assay for Creatine Kinase-MB Isoenzyme

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With a two-site CK-MB assay, we screened serum samples from 1008 blood donors for the presence of antibodies to mouse monoclonal immunoglobulin. These antibodies were capable of cross-linking the labeled antibody with the solid-phase antibody in the two-site assay, thus generating a falsely high apparent CK-MB concentration. In 92 (9.12%) of the blood donors tested, apparent CK-MB concentrations of 10–1000 μg/L decreased to <3 μg/L when re-assayed with non-immune mouse serum (10 mL/L) included in the assay reagent. We tested the ability of non-immune sera from other animal species to lower the concentration of apparent CK-MB in 58 of the 92 samples. Bovine and ovine serum were almost as effective as mouse serum; feline, canine, and rabbit serum were less effective. Of the samples tested, 12% (1.1% of the original population screened) showed apparent CK-MB values that either were not depressed by bovine serum or were only partly depressed. We discuss the possible etiology of these antibodies in normal subjects and recommend that all mouse monoclonal two-site assays should contain non-immune mouse serum (or a suitable “irrelevant” mouse monoclonal antibody) to prevent false-positive results.

**Additional Keyphrases:** tests of other species; analytical error

Several workers have reported interference in immunoassay procedures from circulating antibodies in human serum samples (1–11), antibodies directed either against the substance being measured—e.g., TSH (9)—or against the specific immunoglobulin being used in the assay, usually sheep or rabbit antiserum (e.g., 1, 3, 8). Two-site immunoradiometric assays are particularly prone to interference by antibodies in the second category, because any agent capable of cross-linking the labeled antibody with the antibody on the solid phase can generate a false-positive signal in the absence of antigen (3, 12). The incidence of antibodies directed against sheep or bovine immunoglobulin and capable of interfering in an α-fetoprotein assay in which sheep antiserum is used has been reported to be 7% in a population of blood donors, and was suggested to be due to dietary exposure to cow's milk (3). Another study has suggested an incidence of interference of 0.52% in a labeled-antibody assay for Australia antigen, due to the same cause but involving guinea-pig antiserum (11).

With the advent of monoclonal antibodies and the increasing use of these reagents in labeled-antibody assays, the incidence of circulating antibodies directed against mouse immunoglobulin in particular becomes of practical importance. We have recently developed two-site labeled mouse monoclonal antibody assays for the three cytoplasmic forms of creatine kinase (CK; EC 2.7.3.2): muscle-type CK-MM, heart-type CK-MB, and brain-type CK-BB (13, 14). While using these assays to measure CK in serum in various clinical situations we noted two subjects with abnormally high concentrations of all three isoenzymes (per liter, >300 μg of CK-MB and CK-BB, >1000 μg of CK-MM). In both patients these high values appeared to be ascribable to the presence of circulating antibodies against mouse monoclonal immunoglobulin (see Results). This prompted us to examine the incidence of such circulating antibodies in normal subjects. Therefore we examined serum samples from 1008 blood donors for the presence of these antibodies and, in an effort to determine their possible etiology, we have tested the ability of various animal immunoglobulins to block out the cross-linking of mouse monoclonal antibodies produced by positive serum samples.

**Materials and Methods**

**Animal sera.** Non-immune sera from rabbits, sheep, guinea-pigs, and rats were obtained by bleeding animals from the Central Animal Services, University of Cambridge. Non-immune mouse serum was obtained from Seralab, Crawley Down, Sussex, U.K.; non-immune sera from pigs, dogs, cats, pigeons, and cattle were from DAKO Ltd., High Wycombe, Bucks, U.K. Non-immune horse serum was a gift of Professor R. R. A. Coombs. Sheep anti-mouse antiserum was a gift of Dr. K. Siddell.

**Serum samples from blood donors.** Human sera were obtained from the East Anglian Regional Blood Transfusion Centre, identified as to the age and sex of each donor. Serum samples were divided into aliquots, stored at −70 °C, and thawed immediately before assay.

**Assay procedures.** Two-site labeled antibody assays for CK-MM, CK-MB, and CK-BB were performed exactly as described previously (13, 14). The assay chosen as the test system for the presence of anti-mouse immunoglobulins in human serum samples was the "reverse two-step" version of the CK-MB two-site assay (see Results). We incubated 100 μL of serum sample with 100 μL of labeled anti-B subunit antibody 2AC5 (about 30 000 counts/min in SSV (single-strength Veronal) buffer (13) for 1 h, at room temperature, then added 50 μL of anti-M subunit antibody 15CH2 covalently coupled to magnetic solid phase (13). After shaking this mixture at room temperature for 1 h, we removed the solid phase by magnetic separation, washed, and counted its bound radioactivity as described previously (13). In experiments to test the effects of non-immune animal sera on the assay, we added these sera (to give 10 mL/L) to the buffer containing the first labeled 2AC5 antibody. In experiments testing the effect of sheep anti-mouse serum as a
"model system" on the two-site CK-MB assay (see Figure 2 below) we performed the assay either in the reverse two-step procedure as described above but substituting sheep antiserum for the 100 μL of human serum sample, or as a "forward two-step" procedure. In the latter, 100 μL of diluted antiserum was mixed with 50 μL of 15CH2 solid phase (13), incubated for 1 h, separated, and then incubated for 1 h with 100 μL of 2AC5-labeled antibody (13), separated, and the radioactivity counted. We also performed the assay by a simultaneous procedure in which 100 μL of antiserum, 100 μL of 2AC5-labeled antibody, and 50 μL of 15CH2 solid phase were incubated together for 1 h, then separated and the radioactivity was counted.

Absorption procedures. Positive samples were absorbed by incubation with Protein A-Sepharose (Sigma Chemical Co. Poole, Dorset, U.K.) or with mouse monoclonal antibody X63 covalently attached to finely divided cellulose (a gift of Dr. K. Siddle) overnight at 4 °C. After removing the solid phase by centrifugation, we tested the remaining serum supernate with the reverse two-step CK-MB assay.

Results

Serum samples from two patients (BMW2 and BMW10) who had suffered minor head injuries were initially found to have unusually high concentrations of all three CK isoenzymes (>300 μg/L for CK-MB and CK-BB, >1000 μg/L for CK-MM). Additionally, serial dilution of serum from each patient before assay did not give results for immunoreactivity that paralleled the respective standard curve for the two-site CK-BB assay (Figure 1). The high results for apparent CK-isoenzyme immunoreactivity could be abolished either by pre-absorbing each serum sample with Protein A-Sepharose or by absorbing the sample with a "nonspecific" monoclonal antibody (X63) attached to a solid phase (Table 1). We also could abolish apparent immunoreactivity by including 10 μL of non-immune mouse serum per liter of assay buffer. None of these treatments had any effect on the apparent immunoreactivity of a serum sample to which genuine purified CK-isoenzyme had been added (Table 1). Moreover, the serum samples from patients BMW2 and BMW10 also showed very high immunoreactivity in a two-site mouse monoclonal antibody assay of choriogonadotropin, which also could be abolished by including non-immune mouse serum as described (Dr. T. Gard, personal communication). These experiments suggested that circulating anti-mouse monoclonal immunoglobulin antibodies were present in both patients and were cross-linking the labeled antibody and the solid-phase antibody.

The order of addition of reagents in a two-site labeled antibody assay can be varied in three basic ways (15). The analyte can be removed from solution by a solid-phase antibody and then reacted with a labeled antibody (a "forward" two-step assay), or the analyte can be reacted with a labeled antibody and then removed from solution with a solid-phase antibody (a "reverse" two-step assay), or the labeled antibody and the solid-phase antibody can be added simultaneously and, after a suitable incubation, the two antibody–analyte complex can be removed from solution—a "simultaneous" assay (15). Because it appeared possible that the efficiency with which circulating endogenous antibodies cross-linked the two mouse monoclonal antibodies in each assay could vary with the order of addition of reagents, we tested the effect of polyclonal sheep anti-mouse antiserum (as a model system) on variants of the CK-MB and CK-BB assays. Both the CK-MB and the CK-BB assay were considerably more sensitive to the cross-linking effect of anti-mouse antiserum in the reverse two-step procedure than in the forward two-step procedure (Figure 2). Moreover, the serum samples from both of the above patients showed higher apparent concentrations of CK-BB when the assay was performed in the reverse direction than when in the forward direction. Since the two-site CK-MB assay could be performed more rapidly than the two-site CK-BB assay (2 h vs 5 h), a reverse two-step CK-MB assay with magnetic separation (see Methods) was chosen as the system for screening blood-donor samples.

We tested serum samples from 1008 blood donors in the reverse two-step CK-MB assay. We then reassayed all samples giving apparent CK-MB concentrations exceeding an arbitrarily chosen value of 10 μg/L, using the same procedure except that we included non-immune mouse serum (10 mL/L) in the SSV buffer in which the labeled 2AC5 antibody was added. Of the 1008 samples we tested, 92 (9.12%) showed concentrations >10 μg/L; however, all 92 samples showed apparent CK-MB concentrations of <3 μg/L when reassayed in the presence of non-immune mouse serum. The concentrations of apparent CK-MB seen when

![Diagram](image.png)

Fig. 1. Non-parallel dilution of CK-BB immunoreactivity in BMW2 serum. Performed exactly as described previously (13). &; serum from patient BMW2 included in the assay at increasing dilutions in SSV buffer (13), O; standard curve of assay. Nonspecific binding (not subtracted) was 230 counts/min. All values are the means of duplicate determinations, all agreeing within 5%.

Table 1. Absorption and Blocking Experiments with BMW2 and BMW10 Sera before CK-MB Assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>No pre-incubation</th>
<th>Assay buffer supplemented with non-immune mouse serum, no pre-incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X-63 Protein A-Sepharose</td>
<td></td>
</tr>
<tr>
<td>BMW2</td>
<td>39</td>
<td>6.1, 14</td>
</tr>
<tr>
<td>BMW10</td>
<td>47</td>
<td>2.5, 10</td>
</tr>
<tr>
<td>CK-MB standard</td>
<td>66</td>
<td>66</td>
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Sera from BMW2 and BMW10 were pre-incubated overnight at 4 °C. Results are expressed as percentage binding of labeled 2AC5 antibody (means of duplicates agreeing within 5%). Nonspecific binding was 0.9%.
The pigs by shown SLit. mill. Fig. we other anti-mouse 35 common respects. We investigated the possible etiology of these endogenous anti-mouse immunoglobulin antibodies in normal subjects by testing the ability of non-immune sera from animals other than mice to lower the apparent CK-MB concentrations in positive serum. Of the original 92 positive samples, we reassayed 58, using, per liter, 10 mL of non-immune serum from cattle, sheep, rabbits, cats, dogs, rats, or guineapigs instead of non-immune mouse serum, and 43 of these non-immune mouse serum was omitted from the assay varied from 10 to 1000 μg/L. However, most (68%) values were <60 μg/L. Of the blood-donor samples we assayed, 531 were from men and 477 were from women; 61 (11.4%) of the former and 31 (6.4%) of the latter were "positive" for the presence of anti-mouse monoclonal antibodies—i.e., they showed CK-MB values >10 μg/L, which fell to values of <3 μg/L when reassayed in the presence of 10 mL of non-immune mouse serum per liter.

Figure 3 shows the distribution of apparent CK-MB concentrations produced by these antibodies in men as compared with women. The four highest apparent CK-MB values seen in women were 160, 240, 260, and 400 μg/L, from 38-, 25-, 27-, and 24-year-old women, respectively. The four highest apparent CK-MB values in men were 170, 220, 300, and 1000 μg/L, from 30-, 45-, 21-, and 40-year-old men, respectively. Positive samples were approximately twice as common in men as in women in all age ranges, except for those donors older than 55 years and those between 26 and 35 years, where the incidence was approximately equal.

We investigated the possible etiology of these endogenous anti-mouse immunoglobulin antibodies in normal subjects by testing the ability of non-immune sera from animals other than mice to lower the apparent CK-MB concentrations in positive serum. Of the original 92 positive samples, we reassayed 58, using, per liter, 10 mL of non-immune serum from cattle, sheep, rabbits, cats, dogs, rats, or guineapigs instead of non-immune mouse serum, and 43 of these

![Fig. 2. Effects of sheep anti-mouse antiserum on variants of the CK-MB (left) and CK-BB assays (right) as a model system.](image)

The assays were performed as described previously (13), with total counts of 30 000 cpm added to each assay mixture. Sheep anti-mouse antiserum was diluted to 10 mL in SSV buffer and then serially diluted twofold 14 consecutive times, i.e., the concentration of antiserum added at dilution 1 was 10 mL and at dilution 15 it was 0.61 μL. We then assayed 100-μL aliquots of each dilution in the CK-MB and CK-BB assays, using either the forward (C) or the reverse (I) two-step procedure. All values shown are the means of duplicates agreeing within 5%. Nonspecific binding (100–400 counts/min) has been subtracted in both assays.

![Fig. 3. Distribution of apparent raised CK-MB concentrations according to sex.](image)

Shaded bars: men; clear bars: women.
samples were also tested with non-immune serum from pigeons (Table 2).

While (by definition) non-immune mouse serum produced an 80% decrease in the apparent CK-MB concentration in 100% of the positive samples tested, other animal sera produced a complex picture, with serum from cattle, sheep, rats, and guinea-pigs being almost as effective as that from the mouse, and with cats', dogs, rabbits, and (especially) pigeons being much less effective (Table 2). Serum of the blood-donor samples (representing 1.1% of the original population screened) were 90% blocked by non-immune mouse serum but were either unaffected or only partly blocked by bovine serum. Of these, one was unaffected by cattle, sheep, or rat serum, but was 90% inhibited by mouse or guinea-pig serum. Another was 90% inhibited by mouse, rat, and sheep serum, but only partly inhibited by bovine and guinea-pig serum. In general, serum samples which could be blocked by bovine serum were similarly blocked by ovine serum, except for four samples in which sheep serum was more effective than cattle serum and two samples of which the reverse was true. In general, cat, dog, and rabbit serum produced similar degrees of blocking in each serum sample tested. Guinea-pig and rat serum also produced similar results, except for two samples that were almost completely blocked (>80%) by guinea-pig serum but were essentially unaffected (<15%) by rat serum, and three samples for which rat serum was twice as effective as guinea-pig serum. Pigeon serum produced no effect in 14 of the 43 samples tested and only produced a 30% or greater decrease in the apparent CK-MB concentration in five of the samples tested. In separate experiments we also tested 15 of the 58 positive samples with 10 mL of non-immune horse serum per liter. This produced a decrease of at least 90% in the apparent CK-MB concentration in all 15 samples; however, these samples were also equally well blocked by bovine and ovine serum. None of the non-immune sera we used produced any effect on the standard curve for the reverse two-step CK-MB assay when 10 mL/L was added to the assay buffer.

All the serum specificity test results presented in Table 2 were performed with 10 mL of non-immune animal sera included, per liter, in the buffer containing labeled antibody 2AC5. With mouse serum this sufficed to block even the highest apparent CK-MB concentration found (>1000 μg/L). Ten positive serum samples were further tested with lower dilutions of non-immune mouse and cattle serum. Figure 4 shows the titration curve for one sample in which cattle and mouse serum appeared to be equally effective at 10 mL/L (Figure 4A) and a second sample (Figure 4B) in which cattle serum appeared less effective at lowering the apparent CK-MB concentration than was mouse serum. Figure 4A shows that cattle serum was in fact less effective at 0.1 mL/L than mouse serum, and Figure 4B shows that, although cattle and mouse serum were equally effective at 0.1 mL/L, mouse serum (but not cattle serum) could depress the value for apparent CK-MB even further if increased to 1 mL/L.

Discussion

Several workers have reported interference in immunoadsay procedures by circulating antibodies to animal immunoglobulins (I–II), but these reports have involved polyclonal antisera, and, as far as we are aware, ours is the first study in which mouse monoclonal antibodies were used both as labeled tracer and as solid-phase antibody. Following an early report of interference in an assay for hepatitis-B antigen by circulating antibodies against rabbit immunoglobulin which cross-linked the solid phase with the labeled tracer (I), there have been several reports from neonatal screening programs measuring thyrotropin (2, 5, 6, 11). Falsely high values have been found, apparently because of transplacental acquisition by the neonate of antibodies directed against the animal immunoglobulin used in either radioimmunoassays (e.g., 6) or in immunoradiometric assays for TSH (e.g., 11). Here our initial observations of two patients with very high concentrations of CK isoenzymes showing non-parallel dilution characteristics in each assay (Figure 1), which could be decreased to normal concentrations by the inclusion of non-immune mouse serum or by pre-absorption with Protein A or a mouse monoclonal solid-phase (Table 1), strongly suggest that interfering factor(s)

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Table 2. Effect of Non-Immune Sera from Different Species on Apparent CK-MB Concentrations in Positive Serum Samples

<table>
<thead>
<tr>
<th>Decrease in apparent CK-MB value, %</th>
<th>Percent of total serum samples tested</th>
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<tbody>
<tr>
<td>Mouse</td>
<td>Sheep</td>
</tr>
<tr>
<td>&gt;90</td>
<td></td>
</tr>
<tr>
<td>&gt;80</td>
<td>100</td>
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<td>&gt;60</td>
<td></td>
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<td>&gt;40</td>
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<td>&gt;20</td>
<td></td>
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<tr>
<td>&lt;20</td>
<td></td>
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</table>

*58 high-CK-MB serum samples (43 in the case of pigeon serum) were assayed in duplicate in the reverse two-step CK-MB assay, with or without non-immune sera, from the sources indicated. The final concentration of added serum in the first step of the assay was 5 mL/L. All duplicates agreed within 5%; all results have been rounded to the nearest whole percentage point.
present in the CK assays belong in the same category and represent antibodies against mouse monoclonal immuno-
globulin.

We were surprised to find such a high incidence (9.12%) of such antibodies in a normal population of blood donors. An incidence of approximately 0.51% of anti-guinea-pig immuno-
globulin antibodies in a population of blood donors screened with a hepatitis-B antigen assay has been reported (1), and 14 cases out of 10 261 (i.e., 0.13%) were shown to have anti-rabbit immunoglobulin antibodies in one thyro-
tropin-screening program (5) and seven out of 76 734 (i.e., 0.09%) in another. One case has recently appeared (11) showing in one instance among 16 000 screened with a thyro-
tropin assay in which a labeled mouse monoclonal antibody was used as tracer and a sheep polyclonal antibody as solid phase. A similarly designed assay, but in this instance for the measurement of α-fetoprotein, showed one case among the 300 samples analyzed of a spuriously high concentra-
tion, which could be abolished by the inclusion of either non-
immune mouse or non-immune sheep serum (15). The incidence found here is closer to the value of 7% of Scottish blood donors who were found (2) to have anti-sheep immuno-
globulin antibodies interfering in a polyclonal assay for α-
fetoprotein. Unlike the present study, the overall incidence of these antibodies appeared to be equal between the sexes; here, we find the overall incidence to be greater in men. If one assumes that the apparent CK-MB concentration pro-
duced in the two-site assay reflects the titer of circulating anti-mouse immunoglobulin antibodies, then there is no striking difference between the range of titers found in men as compared with that for women (Figure 3).

Although the overall incidence of 9.12% of a normal population showing these antibodies may appear high, we believe it to be an underestimate, for two reasons. First: we arbitrarily chose a concentration of apparent CK-MB of 10 µg/L when screening for positive blood-donor samples, while the upper limit of normal for true CK-MB in serum is probably <2 µg/L (13). Hence, donor samples with low concentrations of anti-mouse immunoglobulin antibodies—producing values of CK-MB apparently exceeding the normal range but less than 10 µg/L—have been excluded from further study. Secondly: the two-site assay for CK-MB, whether performed in the reverse or the forward direction, has been optimized for the cross linking of labeled antibody 2AC5 and solid-phase antibody 15CH₉ by CK-MB (13) rather than by a third antibody. Such optimization (e.g., by adjusting incubation times) or, as suggested in Figure 2, adoption of the lengthier reverse two-step CK-BB assay (which appears to be more sensitive to low concentrations of anti-mouse immunoglobulin antibodies—see Figure 2) as the screening procedure would possibly have led to a consid-
ernably higher proportion of positive samples.

We are unable to come to any firm conclusions as to the etiology of antibodies in normal subjects reacting with mouse monoclonal immunoglobulin. The presence of antib-
odies to rabbit immunoglobulin has been postulated to be due to injection with either vaccines containing rabbit serum (4, 6) or with rabbit or horse anti-thymocyte globulin (7). Animal handling has also been considered to be a cause (8); possibly cat or dog serum might have been expected to be more effective (Table 2) in the present study if this were an etiological factor here. The 7% incidence of anti-sheep immunoglobulin antibodies in a Scottish blood-donor popu-
lation was postulated to be due to the ingestion of cow’s milk, which gave rise to anti-cattle immunoglobulin anti-

bodies that then cross reacted with sheep immunoglobulin (3). Antibodies to lactalbumin are known to be present in the serum of 30–50% of a normal human population (18, 19) and dietary exposure presumably could similarly immunize against cattle immunoglobulin. While we cannot exclude this as a major etiological factor in the production of antibodies against mouse immunoglobulin, in 12% of our positive samples tested in the present study cattle serum was either ineffective or was much less effective than mouse serum in lowering the apparent CK-MB concentration level (Table 2). Figure 4B suggests a complex picture in which cattle serum contains a subpopulation of immunoglobulin molecules, which can cross react with some but not all of the epitopes involved in the cross-linking effect seen in the CK-
MB assay. In view of the fact that anti-mouse immunoglo-
bulin antibodies may be present more commonly than the present study suggests (see above), it is reasonable to assume that they represent “rheumatoid-like” factors and thus may merely reflect a facet of the normal immune system.

We have systematically tested only for the presence of antibodies to mouse immunoglobulin with a single two-site mouse monoclonal antibody assay, but we assume that the results apply to other similar assays, although it is probable that the apparent incidence of these antibodies will vary with assay design (see Figure 2) and conceivably with the individual pairs of monoclonal antibodies involved. The practical importance of the present finding is that interference in all two-site assays involving monoclonal antibody originating from mice should be blocked by including normal mouse serum or an unrelated mouse monoclonal anti-
body if falsely increased results are to be completely avoided.

The presence of anti-mouse monoclonal immunoglobulin antibodies in normal subjects may also help to explain a discrepancy between the apparent upper limit of the normal range for serum CK-MB as determined with the monoclonal two-site assay—approximately 2 µg/L (13)—and that deter-
mined previously with a two-site polyclonal assay involving a labeled goat anti-CK-MM antiserum and a solid-phase sheep anti-CK-BB antiserum (17). The two assays gave comparable values for CK-MB in serum from patients with myocardial infarction (13, 17), but the polyclonal system suggested an upper limit of normal of approximately 45 µg/L. Because the previous assay was developed at a time when the significance of anti-immunoglobulin antibodies was not fully appreciated and non-immune goat or sheep serum was not included in the assay, it is possible that some of the higher values found in control sera represented apparent CK-MB concentrations produced by the type of endogenous antibody interference we have described here.

We are grateful to Dr. M. Watson for supplying us with the original two sera showing abnormal concentrations of CK isoen-
yzmes. We thank Professor R. R. A. Coombs and Dr. K. Siddell for helpful discussions and Miss H. Wombwell for expert technical assistance. A.P.J. was the holder of a Medical Research Council Scholarship for Training in Research Methods. This work was supported by a grant from the Nuffield Foundation to R.J.T.

References