Preparation of F(ab')2 Fragments of Immunoglobulin G

Jerald J. Killion¹ and Elizabeth M. Holtgrew²

We describe a simple protocol for the preparation of F(ab')2 fragments of immunoglobulin G, based upon the known Fc-binding properties of protein A-Sepharose. The fragment preparations of xenogeneic and allogeneic anti-IgG were nontoxic to intact target cells, and were able to block the cytotoxicity of intact antibody. This method should therefore be useful for fundamental studies not requiring biochemical homogeneity.

Additional Keyphrases: cytotoxicity • xenogeneic and allogeneic serum

The growing use of immunological assays requires rapid, reproducible protocols for reagent preparation. This is especially true in the preparation of fragments from polyvalent and monoclonal antibodies. The classical method of peptidemidediated fragmentation of immunoglobulin G involves several steps, including the isolation of γ-globulin-class serum protein, digestion with enzyme, and final recovery of F(ab')2 fragments of IgG (1). This procedure, although methodologically thorough, requires large volumes of serum and involves as much as a week of multi-step processes. Madsen and Rodkey (2) have described a modification for small volumes of serum routinely obtained from mouse experiments. We outline here a further simplification of the method of Goding (3) for the preparation of F(ab')2 fragments of IgG. Although the use of protein A-Sepharose is a common method for the purification of IgG fragments, we have further simplified the procedure by using the same solution for the peptid digestion of IgG as that used for elution of the IgG from protein A-Sepharose. The method results in recovery of nearly 100% of protein, ready for use in studies requiring an operationally defined fragment, namely: (a) lack of binding to an Fc-specific protein A matrix, (b) the loss of complement-dependent cytotoxicity toward intact target cells, and (c) competitive abrogation by the fragments of whole xenogeneic and allogeneic hyperimmune antisera cytotoxicity.

Materials and Methods

Xenogeneic and allogeneic serum: Two different antisera served as model sera for fragment preparation. Rabbit antimouse thymocyte serum (RoMts) (lot 9468; Cappel Laboratories, Cochranville, PA 19330) was reconstituted with distilled water to 56 mg/mL and stored at −80 °C. Preliminary experiments with B6D2F1 mouse spleen cells (Jackson Laboratories, Bar Harbor, MA 04609) used as target cells indicated that this serum killed at least 50% of the cells when the serum was diluted 512- and 1024-fold. Hyperimmune allogeneic murine serum was prepared by at least four intraperitoneal injections, two weeks apart, of age-matched C57BL/6 mice (haplotype H-2b; Charles River Breeding Laboratories, Wilmington, MA 01887) with 10⁶ spleen and liver cells from DBA/2 mice (haplotype H-2b).

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Table 1. Cytotoxicity of Rabbit Anti-Mouse Thymocyte Serum (RaMTS)

<table>
<thead>
<tr>
<th>Serum or serum fraction</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>512</th>
<th>1024</th>
</tr>
</thead>
<tbody>
<tr>
<td>RaMTS + C(^b)</td>
<td></td>
<td></td>
<td></td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>&gt;95</td>
</tr>
<tr>
<td>RaMTS IgG + C</td>
<td></td>
<td></td>
<td></td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;90</td>
</tr>
<tr>
<td>RaMTS F(ab')(^2) + IgG + C</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

*The cytotoxicity from complement alone was <5%. Target cells were B6D2F1 spleen cells. \(^b\) C = complement. Preparation concentrated to an estimated 10 mg of protein per milliliter.

F(ab')\(^2\): This assay, described in detail elsewhere (6), is a modification of the NIH technique involving trypan blue dye exclusion (7, 8). For assays with whole serum, 10\(^5\) target cells in 50 µL of RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY 14072) containing 100 µL of heat-inactivated fetal bovine serum per liter were incubated with 50 µL of test serum in 96-well microtiter plates for 1 h at 37°C. Trypan blue (25 µL of a 4 g/L solution) was added to the wells and the percent of stained (killed) cells was calculated from counts of at least 200 cells. Blocking assays with F(ab')\(^2\) preparations were performed by prior addition of 50 µL of the fragment preparations to 50 µL of the cell suspension, incubation for 30 min at 37°C, and then proceeding as above after the addition of test serum. All assays included positive and negative controls, and intra-assay variation of replicates was less than 8%. \(^2\)Analysis of cell counts with a minimum of 200 cells required a minimum of 10% difference in the percentage of stained cells to be considered statistically significant at a level of p < 0.05.

Results

Table 1 demonstrates that an aliquot of the same RaMTS serum as that applied to the protein A-Sepharose column was highly cytotoxic to B6D2F1 spleen cells out to a 1024-fold dilution, whereas the F(ab')\(^2\) preparation contained no cytotoxic activity, even at 10 mg/mL (500 µg/10\(^6\) cells in the undiluted well). Moreover, the cytotoxicity of whole serum resided in the IgG fraction of serum. Hence, two criteria of immunoglobulin fragmentation were fulfilled, i.e., the lack of binding to protein A and the loss of cytotoxicity toward intact target cells. As Table 2 shows, the cytotoxicity of whole RaMTS serum was blocked by preincubation of the target cells with the F(ab')\(^2\) preparation at 500, 50, and 5 µg per 10\(^6\) cells, respectively. The whole serum was tested at 100-, 200-, and 400-fold dilutions, each of which resulted in >90% cell kill. Blocking of whole-serum cytotoxicity was observed at 500 and 50 µg per 10\(^6\) cells. Hence, a third criterion of proper fragmentation was met, the ability of the F(ab')\(^2\) fragments to compete for the antigenic site with intact immunoglobulin.

This F(ab')\(^2\) preparation protocol was tested by using a more common model for immunological studies, allogeneric hyperimmune serum. \(\alpha\)-H-2\(^d\) serum was serially diluted from 32- to 128-fold and tested for cytotoxicity against DBA/2 spleen cells that had been preincubated with either 500 µg of fragments per 10\(^6\) cells in an equal volume of PBS. The results are shown in Table 3. The cytotoxicity of the \(\alpha\)-H-2\(^d\) serum ranged from 90 to 93% at a 32-fold dilution to 48-66% at a 128-fold dilution, for a 30-min preincubation of the target cells in PBS. This cytotoxicity was completely abrogated when the \(\alpha\)-H-2\(^d\) F(ab')\(^2\) fragments were used to block antibody binding, the cytotoxicity ranging from 17 to 6% over a 32- to 128-fold serum dilution.

Discussion

The protocol for the preparation of F(ab')\(^2\) fragments described here was motivated by the need to develop blocking assays for use in various immunological studies. We were faced with a limited and sporadic supply of antisera and found that several steps for the classical preparation of F(ab')\(^2\) fragments (7) could be combined or eliminated, because our use of the fragments in immunofluorescent and binding assays did not require homogeneous protein preparations. Analysis of similar fragment preparations by polyacrylamide gel electrophoresis showed that most of the protein was present at a molecular mass near 110 000 Da, presumably the F(ab')\(^2\) fragment (9). Trace amounts of intact IgG and peptic were also detected. It is entirely possible that protein A-Sepharose is not exclusive for the binding and subsequent release of serum immunoglobulins, but the present study was designed solely around the use and detection of immunoglobulin.

We believe that the ease of preparation, the excellent recovery of material, and the quick preparation time (72 h) of this operationally defined F(ab')\(^2\) will aid investigators in need of noncytotoxic yet antigen-binding ligands. This preparation protocol is based upon the Fc-specificity of protein A and the described methodology of Goding (3), by using the advantage gained by eluting the IgG class immunoglobulin at the same pH and volume as used for peptic digestion. The

Table 2. Blocking of Cytotoxicity of RaMTS by F(ab')\(^2\) Fragments

<table>
<thead>
<tr>
<th>Serum and fragments</th>
<th>100</th>
<th>200</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td>RaMTS + PBS + C</td>
<td>98</td>
<td>98</td>
<td>91</td>
</tr>
<tr>
<td>RaMTS + 500 µg of F(ab')(^2) + C</td>
<td>26 = 2(^a)</td>
<td>30 ± 2(^b)</td>
<td>12 ± 7(^b)</td>
</tr>
<tr>
<td>RaMTS + 50 µg of F(ab')(^2) + C</td>
<td>44 ± 9(^b)</td>
<td>28 ± 6(^b)</td>
<td>11 ± 3(^b)</td>
</tr>
<tr>
<td>RaMTS + 5 µg of F(ab')(^2) + C</td>
<td>96 ± 2</td>
<td>94 ± 1</td>
<td>66 ± 23</td>
</tr>
</tbody>
</table>

*The cytotoxicity from complement (C) alone was <5%. Target cells were B6D2F1 spleen cells. Values are the mean ± SD of duplicates.

*Statistically fewer (p < 0.05) stained cells than that observed for either the positive control (RaMTS + PBS + C) or the RaMTS + 5 µg of F(ab')\(^2\) + C.

Table 3. Blocking of Cytotoxicity of C57BL/6 Anti-DBA/2 Serum (\(\alpha\)-H-2\(^d\)) by F(ab')\(^2\) IgG Fragments

<table>
<thead>
<tr>
<th>Serum and fragments</th>
<th>1</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>512</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)-H-2(^d) + PBS + C</td>
<td></td>
<td>93 ± 2</td>
<td>64 ± 1</td>
<td>66 ± 11</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>(\alpha)-H-2(^d) + 300 µg of F(ab')(^2) + C</td>
<td>17 ± 6(^b)</td>
<td>10 ± 11(^b)</td>
<td>8 ± 4(^b)</td>
<td>10</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

*The cytotoxicity from complement (C) alone was <5%. Target cells were DBA/2 spleen cells. Values are the mean ± SD of duplicates.

*Statistically fewer (p < 0.05) stained cells than that observed for \(\alpha\)-H-2\(^d\) + PBS + C.
retention of antigen affinity has been demonstrated and the protocol described here should be useful where strict protein homogeneity is not required.

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References

Changes in Serum Iron and Leukocyte Count Associated with Open-Heart Surgery

Edward J. Fitzsimons1 and Garth H. Ballantyne2

Rapid and pronounced changes in serum iron concentration and leukocyte count in association with open-heart surgery were observed in each of 58 patients. We examined the temporal aspects of these alterations. An initial increase in Fe concentration from a mean of 0.94 mg/L before surgery to 1.20 mg/L was observed within 6 h of the start of surgery. Decreased Fe concentration, a phenomenon previously associated with physiologically stressful events, became apparent 12 h after surgery, by which time the mean Fe concentration had declined to 0.26 mg/L. An increase in the mean leukocyte count, from 7.1 to 15.2 × 1000/mm3 was observed within 6 h of the start of surgery. An increase in ferritin concentration in serum was concurrent with decreased iron concentration.

Additional Keyphrases: concomitants of surgical stress • ferritin concentration, changes with time

Serum iron has been determined as part of a multiple analysis profile done with the SMAC (Sequential Multiple Analyzer with Computer; Technicon Instruments Corp., Tarrytown, NY 10591) at Northwestern Memorial Hospital since May 1977. A review of the clinical chemistry reports for patients admitted to this hospital revealed that many showed a pronounced decrease in their iron concentration in serum. This decrease has been observed in association with acute myocardial infarction (1) and with other physiological stressful conditions such as surgical, infectious, neurologic, and obstetric events (2). The decrease reportedly (1, 2) occurs within 24 to 48 h of the stressing event, together with an increase in the leukocyte count.

Here we describe more precisely the temporal aspects of these changes. To do so, we reviewed the laboratory results for patients admitted to this hospital for open-heart surgery. Such patients routinely have blood drawn for laboratory tests before and within 6 h after the start of surgery, every 6 h for 24 h after surgery, and daily during the rest of the hospital stay.

Materials and Methods

For this study we selected, without conscious bias, 58 patients having open-heart surgery between January 1978 and November 1979. Of these, 15 required valve replacement and 43 had coronary artery bypass grafting. Cardiopulmonary bypass was utilized, and for autotransfusion of sequestered blood and blood salvaged during surgery a Haemonetics Cell Saver (Haemonetics Corp., Natick, MA) was used (3). None of the 58 patients required transfusions of blood from the blood bank. Bypass time ranged from 1 to 3.5 h.

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