A Plaque Assay to Enumerate Circulating Ig-Secreting Cells of Each Type of the Different Ig Classes

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A new method to quantitate circulating cells secreting immunoglobulin (Ig) of each type of the different classes by hemolytic plaque technique is reported. The ratio of kappa (κ)-secreting cells and lambda(λ)-secreting cells of the different Ig classes in normal individuals is almost equal to that of κ:λ light chains in corresponding serum Ig class, whereas the ratio in plasma cell myeloma patients is extraordinarily high or low, indicative of the type and class specificity of this assay system as well as the monoclonal nature of circulating Ig-secreting cells in such patients.

Increased circulating monoclonal B lymphocytes in plasma cell myeloma patients have been shown by immunofluorescence study with the use of either idiotypic antisera directed against the patient's own serum myeloma protein or monospecific antisera directed against heavy and light chains of human Ig.

Recently, we found that IgG plasma cell myeloma patients had increased circulating IgG-secreting cells. Assuming that the increased circulating IgG-secreting cells would represent a circulating compartment of a myeloma cell clone, we undertook the experiments to demonstrate the light chain type of Ig-secreting cells by hemolytic plaque method.

Reverse hemolytic plaque assay (RHPA) developed by Molinaro et al. and by Eby et al. enables us to enumerate even a very small number of Ig-secreting cells intermingled with a large population of lymphocytes as plaque-forming cells (PFC).

Gronowicz et al. reported a quite sophisticated method using RHPA to enumerate all murine plasmacytoma cells secreting Ig of a given type or class. However, their method cannot tell the class and type of each Ig-secreting cell at the same time.

This article describes a method to demonstrate the light chain type of Ig-secreting cells of different Ig classes as PFC, which was made possible simply by changing the combinations of antibodies used in RHPA and also presents data on the number of circulating κ- and λ-secreting cells of each Ig class contained in 10^5 peripheral blood mononuclear cells (PBM) obtained from normal individuals and patients with plasma cell dyscrasias.

MATERIALS AND METHODS

Preparation of κ and λ Light Chains, IgG, IgA, and IgM

Kappa and λ light chains were purified from urinary Bence-Jones protein of respective type obtained from patients with IgD myeloma or Bence-Jones type myeloma to avoid any heavy chain contamination present in other patients with conventional monoclonal IgG or IgA myeloma. The procedures included repeated precipitation with saturated ammonium sulfate and subsequent fractionation by ion-exchange chromatography through 2.5 x 50 cm column of DEAE cellulose (DE23, Whatman Ltd.) and a protein peak of light chain was obtained in fall-through fractions with 0.1 M Tris-phosphate buffer (pH 8.5). After concentration by ultratitration through a PM 10 membrane (Amicon Far East Ltd.), this preparation yielded a single band with rabbit anti-human Ig antiserum and with either anti-human κ antiserum or anti-human λ antiserum on immunoelectrophoresis.

Human IgG was purified from the sera obtained from a normal healthy individual by saturated ammonium sulfate precipitation followed by ion-exchange chromatography through 2.5 x 50 cm column of DEAE cellulose. A protein peak of IgG obtained in fall-through fractions with 0.1 M Tris-phosphate buffer (pH 8.5) was concentrated as above.

Human IgA and IgM were purified from the sera obtained from patients with IgA myeloma or Waldenström's macroglobulinemia by saturated ammonium sulfate precipitation followed by gel filtration through 1.5 x 90 cm Sephacryl S-300 (Pharmacia Fine Chemicals AB) column equilibrated and eluted with 0.02 M Tris-HCl-1.0 M NaCl buffer (pH 8.0). Fractions that formed a precipitin line on Ouchterlony double immunodiffusion only with either rabbit anti-human IgA antiserum or anti-human IgM antiserum were collected and concentrated as above.

Human κ and λ light chains, IgG, IgA, and IgM thus obtained were covalently linked independently to CNBr-activated Sepharose 4B immunoadsorbents (Pharmacia Fine Chemicals AB) and were used to adsorb rabbit anti-κ, anti-λ, anti-IgG, anti-IgA, and anti-IgM, respectively.

Preparation of Anti-κ, Anti-λ, Anti-IgG, Anti-IgA, and Anti-IgM

Rabbits were immunized with a series of three triweekly subcutaneous injections of either κ or λ light chains, IgG, IgA, or IgM preparations (1 mg each in 0.15 M NaCl) emulsified in complete Freund's adjuvant. A week after the last immunization, each antiserum was collected and was fractionated by affinity chromatography over 0.9 x 30 cm CNBr-activated Sepharose 4B column coupled with appropriate antigen. The column was first washed with phosphate buffer-0.5 M NaCl (pH 7.9) and then each adsorbed antise-
agarose in HBSS. After the gelification of the top layer, dishes were Ouchterlony double immunodiffusion.

Preparation of Peripheral Blood Mononuclear Cells (PBM)

PBM were separated from heparinized venous blood by the use of Ficoll-Hypaque density gradient technique. PBM were washed twice with phosphate-buffered saline (PBS), then once with Hank's balanced salt solution (HBSS), and finally resuspended in HBSS.

Reverse Hemolytic Plaque Assay (RHPA)

Light chain type of Ig-secreting cells was demonstrated by using RHPA. Briefly, 0.1 ml of each purified antiserum (10 mg/ml in PBS) as "coupler" was incubated with 0.5 ml of washed and packed sheep erythrocytes (SRBC) and 0.5 ml of CrCl3 (1 mg/ml in 0.15 M NaCl) at 37°C for 1 hr with continuous shaking. One-tenth milliliter of PBM suspension in HBSS and 0.1 ml of 10% v/v suspension in 0.15 M NaCl of freshly antiserum-coupled SRBC were mixed well with 0.8 ml of 0.8% agarose in HBSS kept at 45°C and were plated on 60-mm Petri dishes precoated with 4 ml of 0.8% agarose in HBSS. After the gelification of the top layer, dishes were first incubated at 37°C for 1 hr in a humidified atmosphere, being followed by the addition of 1 ml of "developer" and by further incubation for another 1 hr. After decanting the "developer," dishes were incubated for 45 min with 1 ml of 10% diluted guinea pig serum (Kyokuto Pharmaceutical Industries, #80742) previously absorbed with SRBC as a complement source, and hemolytic plaques were counted. All data represent the mean of duplicate determinations and are expressed as PFC/10^5 PBM.

RESULTS

To demonstrate a light chain type of each IgG-secreting cell in PBM obtained from normal individu-
cells as well as IgMκ- and IgMλ-secreting cells contained in 10^7 PBM of normal individuals were enumerated using purified anti-IgA and anti-IgG as “developer,” respectively, instead of anti-IgG. As seen in Table 2, the sum of the numbers of κ- and λ-secreting cells of IgA and IgM in each normal individual is within the range of the reported numbers of IgA- and IgM-secreting cells by others. In addition, the mean ratio of IgAκ- and IgAλ-secreting cells and that of IgMκ- and IgMλ-secreting cells were quite consistent with κ:λ ratio of the serum IgA and IgM in Japanese, respectively (IgA κ:λ = 0.82:1, IgM κ:λ = 0.93:1).

We then enumerated circulating cells secreting Ig of each type of the different classes in 10^7 PBM obtained from patients with plasma cell dyscrasia. As shown in Table 3, in IgGκ myeloma patients IgGκ-secreting cells are disproportionately increased in number, and in IgGλ myeloma patients, IgGλ-secreting cells are likewise increased, leaving the counterpart of the same Ig class and κ- and λ-secreting cells of other Ig classes within the normal range.

In one patient with Waldenström’s macroglobulinemia, an increase of circulating cells secreting M-protein, as was noted in IgG and IgA myeloma patients, was not remarkable, though the morphologically identified atypical lymphocytes exceeded 40% of PBM. However, the ratio of IgMκ- and IgMλ-secreting cells was out of the range of normal individuals.

**DISCUSSION**

The RHPA that makes it possible to detect individual Ig-secreting cells uses antibody specific for the various Ig types and classes. Therefore, to enumerate PBM secreting Ig of a given type as PFC, we applied RHPA using specific anti-light-chain antibody. However, the combination of anti-light-chain antibody as both “coupler” and “developer” gave no PFC, whereas the combination of anti-light-chain antibody as “coupler” and anti-IgG antibody as “developer” did yield hemolytic plaques. Assuming that PFC appearing in this combination would represent cells secreting each light chain bound to whole IgG because anti-IgG antibody was employed as “developer,” we next enumerated circulating Ig-secreting cells contained in 10^7 PBM of normal individuals using the same combination of antibodies. The results obtained supported our assumption because (1) the sum of the numbers of κ- and λ-secreting cells in each individual approximated the mean value of IgG-secreting cells reported by Shimizu et al.7 and others,11 and (2) the mean ratio of κ- and λ-secreting cells was almost equal to that of κ:λ of the serum IgG in Japanese.

By the experiments using anti-IgA and anti-IgM antibodies as “developer” instead of anti-IgG, we ascertained that we could enumerate IgAκ-, IgAλ-, IgMκ-, and IgMλ-secreting cells as well.

The precise mechanism for the success of our method for enumeration of PBM secreting Ig of a given type of the different classes—namely that the anti-light-chain antibody needed to be coupled to the erythrocytes first before developing reagent was overlaid—is subject to further study. From the results of our work to date we are led to suspect that antilight-chain antibody as “developer” could not bind to light chains bound to secreted Ig because coupling antibody had already occupied them and/or because of the steric change within Ig after the reaction between the coupled erythrocytes and the secreted Ig.

Based on these results, we quantitated Ig-secreting cells in 10^7 PBM obtained from patients with plasma cell dyscrasia using anti-light-chain antibody as “coupler” and anti-IgG, anti-IgA, and anti-IgM as “developer” and found a disproportionate increase of circulating cells secreting Ig of the same type and class as the myeloma protein in such patients. These findings imply (1) the monospecificity of our method concerning the detection and enumeration of PBM secreting Ig of a given type and class, and (2) the monoclonal nature of increased circulating Ig-secreting cells in patients with plasma cell myeloma.

However, such increase of Ig-secreting cells as seen in myeloma patients was not remarkable in the patient

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**Table 3. The Number of κ- and λ- Secretory Cells of Different Ig Classes in 10^7 PBM Obtained From Patients With Plasma Cell Dyscrasia**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Dx*</th>
<th>Class and Type</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>κ</td>
<td>λ</td>
<td>κλ</td>
</tr>
<tr>
<td>1. F.N.</td>
<td>PCM</td>
<td>IgGκ</td>
<td>3,400</td>
<td>21</td>
<td>3,421</td>
</tr>
<tr>
<td>2. M.B.</td>
<td>PCM</td>
<td>IgGκ</td>
<td>440</td>
<td>25</td>
<td>465</td>
</tr>
<tr>
<td>3. M.Y.</td>
<td>PCM</td>
<td>IgGλ</td>
<td>26</td>
<td>770</td>
<td>796</td>
</tr>
<tr>
<td>4. M.W.</td>
<td>PCM</td>
<td>IgAκ</td>
<td>13</td>
<td>11</td>
<td>24</td>
</tr>
<tr>
<td>5. J.M.</td>
<td>PCM</td>
<td>IgAλ</td>
<td>22</td>
<td>20</td>
<td>42</td>
</tr>
<tr>
<td>6. Y.F.</td>
<td>WM</td>
<td>IgMκ</td>
<td>37</td>
<td>30</td>
<td>67</td>
</tr>
</tbody>
</table>

*Dx, diagnosis.
†PCM, plasma cell myeloma.
‡WM, Waldenström’s macroglobulinemia.
with Waldenström's macroglobulinemia. This particular finding may point to a functional difference between plasma cells that secrete Ig vigorously in plasma cell myeloma and cells that have characteristics more like lymphocytes than plasma cells in Waldenström's macroglobulinemia.

In conclusion, our method would be beneficial when studying the monoclonal nature of Ig-secreting cells even if they were not increased in number, and thus would help us reevaluate a wide variety of immunoproliferative disorders as well as other diseases where shifts of ratios of circulating Ig-secreting cells might also be present, but not demonstrable by concurrent presence of monoclonal bands in the serum.

REFERENCES

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