Quantitative Determination of Serum Immunoglobulins in Various Diseases

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By immunoelectrophoresis at least three immunoglobulins, IgG, IgA and IgM can be recognized but quantitative determination of each immunoglobulin has been hampered by methodological difficulties. Recently, however, Fahey and McKelvey described in details an antibody-agar plate method for this purpose. While the author was following their method, it was found that less antiserum was required by spreading antiserum on the agar plate than by mixing it with the agar at 56°C. The mixing method required two or three times the amount of antiserum needed for the spreading method.

By the antibody-agar plate method normal serum IgG, IgA and IgM value were calculated as 18.5±3.9 mg/ml, 3.6±0.68 mg/ml and 1.3±0.37 mg/ml, respectively. These normal values for IgG and for IgA are higher than those reported by Fahey and McKelvey.

The serum IgG, IgA and IgM concentration in various diseases were examined and compared with the total protein concentration. The immunoglobulin levels were not necessarily related to the total protein levels. There was characteristic increase of IgG with decrease of IgA and IgM in G multiple myeloma. All immunoglobulins tended to be decreased in cases under long-term steroid treatment. As for immunoglobulin concentration in other conditions, further studies seem to be required to conclude anything definite.

It is immunoelectrophoretically well established that there are at least three components in the conventional gammaglobulin region. Heremans\(^1\) designated these three components as immunoglobulins because of their ability to become antibodies. These are now known as IgG, IgA and IgM.\(^2\) Furthermore, the fourth immunoglobulin was recently identified and designated as IgD.\(^3\)

It is possible to estimate the serum concentration of each immunoglobulin by thickness of its precipitin line on immunoelectrophoresis plates, but this is still a qualitative rather than a quantitative method. Although West \textit{et al.}\(^4\) described a quantitative determination of IgA and IgM by immunoelectrophoretic analysis, this method seems rather complicated and requires considerable amount of antiserum. For practical purpose an easier method for the quantitation of immunoglobulins has been desired.

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The principle of single diffusion in agar has begun to be used for quantitation of immunoglobulins. Recently Fahey and Mc Kelvey described in details the antibody-agar plate method for the quantitative determination of immunoglobulins.

While the author was following Fahey and Mc Kelvey's method for the preparation of antibody-agar plates, it was found that less antiserum was required by spreading the antiserum on the surface of the agar plate than by mixing the antiserum into the agar at 56°C.

It is the purpose of the present paper to describe a simple method for the determination of serum IgG, IgA and IgM and also to report their levels in various diseases.

MATERIALS AND METHODS

Antiserums: Goat antiserum to human IgG (list no. 71–201), goat antiserum to human IgA (list no. 71–212), goat antiserum to human IgM (list no. 71–211) and horse antiserum to whole human serum (list no. 70–040) were purchased from Hyland Laboratories, Los Angeles, California. The anti-IgG, anti-IgA and anti-IgM gave a single precipitin line in immunoelectrophoresis using normal human serum as antigen (Figs. 1 and 2), while anti-human serum produced multiple lines with the same antigen (Fig. 3). Further specification of the purchased antiserums by absorption was not performed.

Fig. 1. Immunoelectrophoresis of normal human serum.
   a: Specific goat antiserum against human IgG.
   b: Specific goat antiserum against human IgA.

Fig. 2. Immunoelectrophoresis of normal human serum.
   a: Specific goat antiserum against human IgG.
   b: Specific goat antiserum against human IgM.
Fig. 3. Immunoelectrophoresis of human gammaglobulin (F-II) and normal human serum.

a: Human gammaglobulin (Cohn Fraction II).
b: Horse antiserum against whole human serum.
c: Normal human serum.

**Experimental procedure:** Total serum protein was measured by a refractometer (Hitachi) and immunoelectrophoresis was performed by the micromethod as described by Peetoom.8

Two ways were employed for the preparation of antibody-agar plates. The one is essentially the method described by Fahey and McKelvey. One per cent Noble agar (Difco Inc., Detroit, Michigan) in veronal buffer, pH 8.4, ionic strength 0.05, was mixed with the specific antiserum at 56°C and poured to a suitable container on the horizontal surface. The optimal amount of the specific antiserum was decided after a series of trials using different amounts of the antiserum.

The other is a slight modification of the method described above. For this, 1% Noble agar in the veronal buffer without any antiserum was boiled and poured on the horizontal surface of a Petri dish or a plastic container with transparent horizontal bottom. The amount of the agar solution was precalculated so that a uniform layer of 2.5 mm depth was obtained. The agar was allowed to harden at room temperature. The specific antiserum was diluted to twice the original volume with normal saline and poured onto the surface of the agar plate. The quantity of the antiserum required was determined after a series of trials as described later in the section of the results. By shaking the container gently the antiserum solution was spread uniformly on the surface. A dull glass rod or bottom of a test tube was used to bring the antiserum in all directions, if necessary. Unless the atmosphere is too humid, the surface of the agar plate can be dried in 10 minutes so that no liquid movement of the antiserum solution is observed by tilting the container.

After preparation of the plate by either method described above, holes were punched, 2.5 mm in diameter, and 1.5–2.0 cm apart, and the test samples were put into each hole. Care was taken not to overflow the sample, because the volume of each sample should be equal. The plate was incubated at room temperature for 16–48 hours, and diameters of precipitin rings were measured by a scale. When precipitin rings were somewhat indistinct, the agar plates were
Fig. 4. Radial diffusion in an anti-IgG-agar plate after 24 hours of incubation. The specific antiserum to IgG was spread on the agar surface. The plate was stained by amido-black. The double rings were produced by multiple myeloma cases probably due to two kinds of IgG, normal and abnormal.

Fig. 5. Radial diffusion of IgG (top), IgA (middle), IgM (bottom) after 16 hours of incubation.
stained by amido-black with the same method as used for precipitin lines of immunoelectrophoresis (Figs. 4 and 5).

Reference samples: Reference standards for each immunoglobulin were obtained from Hyland Laboratories, Los Angeles, California (lot no. 7010A2B, 7020A2B, and 7030A2B). When any serum contained a high level of IgG, IgA or IgM as compared with the above standards, this serum and six samples of its two-fold dilutions were used for each batch of tests to prepare a graph for each plate comparing the ring diameter to the logarithm of the protein concentration. From this graph the value of unknown samples was determined.

Clinical materials: Serums were obtained from various diseases, but special attention was paid to hematological disorders. The patients were mainly inpatients at Prof. Torikai's Dept. of Medicine, Tohoku University Hospital, Sendai, but cases from other institutions also were included.

Normal control serums were derived from 20 healthy blood donors aged from 15 to 35 years.

Serums were stored at −20°C for one to six months until the assay.

RESULTS

1. Experimental procedure

It was important to precalculate the potency of each purchased antiserum with use of a small Petri dish before making a large antibody-agar plate for the determination of many test samples. Otherwise expensive antiserums may be wasted. As the concentration of antiserum in the agar or its amount on the agar surface decreases, the diameter of the diffusion rings increased until finally they became indistinct even with staining.

It was found that less antiserum was required in the spreading method than in the mixing method, at least when the antibody-agar plates were used soon after the preparation. In the spreading method approximately 0.7 ml of the antiserum was required per 100 cm² of the agar surface. In the mixing method the amount of antiserum had to be increased with the depth of the agar plate. When the antibody-agar plate of 100 cm² surface was made by the mixing method, for the depth of 1.0 mm, 0.7 ml of the antiserum was required, and for the depth of 2.0 or 3.0 mm the antiserum had to be increased two or three times, respectively.

Since the depth of each hole must be at least 2.0 mm so that the amount of test samples may be practically equal, the spreading method was employed to determine immunoglobulin levels throughout this study for economical reason and convenience.

Concerning the standard graph it was nearly straight at 16 hours of incubation at room temperature, but became curved with further diffusion (Fig. 6).
Fig. 6. IgG standard curve. This graph was made by plotting the ring diameter on the abscissa and IgG concentration on the ordinate.

### TABLE 1. Serum immunoglobulin levels from 20 normal persons determined by the agar ring diffusion

<table>
<thead>
<tr>
<th>Authors</th>
<th>IgG (mg/ml)</th>
<th>IgA (mg/ml)</th>
<th>IgM (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fahey &amp; McKelvey</td>
<td>12.4</td>
<td>2.2</td>
<td>2.8</td>
</tr>
<tr>
<td>Present author</td>
<td>18.5</td>
<td>3.9</td>
<td>3.6</td>
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*S.D.: Standard deviation.

phenomenon was most marked with IgG and less with IgM, IgA being intermediate.

2. **Normal controls**

The mean and standard deviation of immunoglobulin values from 20 normal persons are listed and compared with those reported by Fahey and McKelvey in Table 1.
3. **Various diseases**

The levels of IgG, IgA and IgM for serums from various diseases are shown in Figs. 7, 8 and 9. The total protein levels for these serums are also shown in Fig. 10 for comparison.

All multiple myeloma serums with a single exception showed the characteristic increase of IgG and significant decrease of IgA and IgM. In immunoelectrophoresis all these serums had an abnormal IgG precipitin line. Total protein levels of these serums were increased except for one.

In leukemia and in malignant lymphoma no constant pattern of immunoglobulin levels was observed. In these conditions total protein tended to be decreased.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
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<th>mg/ml</th>
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<tr>
<td></td>
<td>40</td>
<td>60</td>
<td>80</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
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<td></td>
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<tr>
<td>MoL</td>
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<td>●</td>
<td></td>
</tr>
<tr>
<td>CML</td>
<td>●</td>
<td>●</td>
<td></td>
</tr>
<tr>
<td>Multiple</td>
<td>●</td>
<td></td>
<td></td>
</tr>
<tr>
<td>myeloma</td>
<td>●</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignant</td>
<td>●</td>
<td>●</td>
<td></td>
</tr>
<tr>
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</tr>
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<td></td>
<td></td>
</tr>
<tr>
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<td>●</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Weber-Christian's disease</td>
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<td>●</td>
<td></td>
</tr>
<tr>
<td>Infectious mononucleosis</td>
<td>●</td>
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Fig. 7. Serum IgG concentration in various diseases. An open circle means a case under the long-term treatment of considerable dose of steroid. "Mean" is the mean value of 20 normal persons.

Fig. 7.-10.

ALL: Acute lymphocytic leukemia  AML: Acute myelocytic leukemia
MoL: Monocytic leukemia  CML: Chronic myelocytic leukemia
Fig. 8. Serum IgA concentration in various diseases. An open circle and "Mean" denotes the same as in Fig. 7.

In hypergammaglobulinemia of unknown cause all three immunoglobulins, especially IgG and IgA, were increased. No abnormal precipitin line was observed in immunoelectrophoresis except for considerable thickening of immunoglobulin lines compared to normal serum. The symptoms of these two cases were similar to those of systemic lupus erythematosus except for the absence of LE cells.

Among cases with infection, one case of pulmonary tuberculosis showed a marked increase of all immunoglobulins, and the cases of infectious hepatitis showed an increase of IgM with normal IgG and IgA level.

In systemic lupus erythematosus, the case without treatment exhibited an increase of all immunoglobulins, but the case under the long-term steroid treatment showed their decrease.

A case of pulseless disease showed increase of all immunoglobulins, especially of IgA and IgM.

In the case of Weber-Christian’s diseases, all immunoglobulins were decreased.
while the total protein was not decreased. This case also had been under the long-term steroid treatment.

In one case of infectious mononucleosis, IgM was elevated, while other immunoglobulins were normal. The other case of infectious mononucleosis had a low heterophil antibody titer.

In this study it was revealed that total protein levels did not necessarily correspond with immunoglobulin levels, but there is always the possibility that chronic protein loss may lower immunoglobulin levels as well as total protein value.

### DISCUSSION

The merit of the radial diffusion technique in the antibody-agar plate for quantitation of immunoglobulins lies in its simplicity and convenience compared with other techniques of immunoglobulin determination. If a specific antiserum is available for any serum antigen, such as lipoprotein, transferrin or various hormones and if they precipitate with their specific antiserums, their quantitation

\[
\begin{array}{|c|c|c|c|c|c|c|}
\hline
\text{Disease} & \text{Mean} & \text{IgM} & \text{mg/ml} \\
\hline
\text{ALL} & \bullet & \bullet & \bullet \\
\text{AML} & \bullet & \bullet & \bullet \\
\text{CML} & \bullet & \bullet & \bullet \\
\text{Multiple myeloma} & \bullet & \bullet & \bullet \\
\text{Malignant lymphoma} & \bullet & \bullet & \bullet \\
\text{Anaplastic lymphoma} & \bullet & \bullet & \bullet \\
\text{Hypergammaglobulinemia, cause unknown} & \bullet & \bullet & \bullet \\
\text{Infection} & \bullet & \bullet & \bullet \\
\text{Pyogenic} & \bullet & \bullet & \bullet \\
\text{Hepatitis} & \bullet & \bullet & \bullet \\
\text{Infect. mononucl.} & \bullet & \bullet & \bullet \\
\text{Systemic lupus erythematosus} & \bullet & \bullet & \bullet \\
\text{Pulseless disease} & \bullet & \bullet & \bullet \\
\text{Weber–Christian’s disease} & \bullet & \bullet & \bullet \\
\text{Infectious mononucleosis} & \bullet & \bullet & \bullet \\
\hline
\end{array}
\]
may similarly be done as well as immunoglobulins. The disadvantage of this method is, however, a minimal increase of the ring diameters with increasing amounts of an antigen. In the case of a large precipitin ring the difference of one millimeter means a significant difference in concentration, and this fact may contribute occasionally to errors of the obtained value. One way to avoid this may be to dilute a test sample with an equal amount of normal saline, when the sample was found to contain a high level of the antigen to be examined, and to double the obtained value afterwards. Deformity of a well or unevenness of antibody in or on the agar plate also may contribute to sources of error. Nevertheless, this technique seems to be a simple and dependable method for the quantitation of serum immunoglobulins.

In this study it was assumed that so long as anti-IgG, anti-IgA or anti-IgM gives a single precipitin line to whole human serum corresponding to its specific region in immunoelectrophoresis, it precipitates only with its specific protein. This seems valid since the original start for dividing the conventional gamma-globulin into G, A and M was based on three different lines in immuno-
Quantitative Determination of Serum Immunoglobulins

Electrophoresis (Fig. 3). Observations were described, however, concerning the cross-reactions among immunoglobulins. Even so, cross-reactions may be minimal, since in this study almost all multiple myeloma cases gave decreased IgA and IgM levels in spite of significant increase of IgG levels. If significant amount of cross-reactions occurred, IgA or IgM also would be found to be increased in these cases. Therefore, for clinical purpose the purchased specific antiserum may be used without further complicated absorption procedures, provided that it gives a single line against whole human serum in immunoelectrophoresis.

On the contrary it should be added that there is no assurance that the specific antiserum employed reacts with all the corresponding immunoglobulin. Fig. 11 reveals that there is IgG which reacts with anti-whole human serum but not with the specific anti-IgG.

Fig. 11. Immunoelectrophoresis of a multiple myeloma serum
a: Antiserum against human IgG.
b: Serum of a multiple myeloma case.
c: Antiserum against whole human serum.

The spreading method is not only economical, but also convenient. Meticulous care should be taken not to overheat the antiserum in the case of the mixing method. Although it has not been reported that exposure of antiserum to 56°C changes its potency, there is evidence that there is decrease of IgM in serum, when serum is incubated at 56°C for thirty minutes. As for the evenness of antibody mixture the mixing method may be superior, but the factor which contributes most to the deformity of the precipitin ring seems to be a disfigure of the sample hole rather than uneven distribution of antiserum in the agar. Afonso described a method of quantitative immunoelectrophoresis, in which antiserum was spread on the surface after electrophoresis of human serum was performed in the agar. Only the surface method can be used if electrophoresis of a test sample should be desired.

If purified samples of known concentration are available for each immunoglobulin, they will be ideal as reference proteins, but since these are difficult to obtain, commercial standards were employed in this study. There is considerable discrepancy in normal values between this study and that of Fahey and McKelvey. It remains to be answered whether this is due to technical or racial difference.
In multiple myeloma cases of this study, it is characteristic that IgG is markedly elevated, while IgA and IgM are decreased. Although gamma A multiple myeloma serum and macroglobulinemia Waldenström serum were not available in this study, it has been reported that there is monoclonal increase of IgA and IgM in gamma A multiple myeloma and in macroglobulinemia Waldenström, respectively, with decrease of other immunoglobulins. This phenomenon can be explained by monoclonal production of immunoglobulins by neoplastic cells. It should be mentioned, however, that monoclonal immunoglobulin abnormality can be present without increase of immunoglobulins, as is shown in one case of multiple myeloma in this study. Furthermore, a case with abnormal monoclonal IgM and without increase of macroglobulin concentration was reported from our laboratory.

In other diseases it is hardly possible to say that there is characteristic increase or decrease of any immunoglobulin. However, it was noted that the cases under long-term steroid treatment tended to have decreased level of all immunoglobulins. In this study the number of cases was not sufficient to derive any definite conclusion and further studies should be carried out to clarify the problem.

The reports on immunoglobulin levels in various diseases have just begun to appear. Lee noted increase of IgM in viral hepatitis. Also, in infectious mononucleosis IgM increase was observed by Wollheim and Williams. Whether the determination of immunoglobulin levels becomes an important tool for diagnosis awaits further studies.

Acknowledgments

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