An In Vitro Technic for Quantitating and Studying the Dynamics of Leukocyte Agglutination

By JOHN D. HARTMAN

In recent years there has developed a growing interest in the phenomenon of leukocyte agglutination. The phenomenon has been studied by many in relationship to agranulocytosis,1,6 and by the author in relationship to hypersensitivity mechanisms.7 Since advancement in knowledge of leukocyte agglutination is dependent upon quantitative technics for measuring the phenomenon, the following technic, with which it is possible to detect microgram quantities of agglutinating agents, is described.

Materials and Methods

Procedure. All solutions should be pyrogen free.5,9 Nine parts of blood are drawn into a syringe containing one part of a saline-heparin (Liquaemin sodium, Organon) solution of such strength to give a final concentration of .03 mg. heparin/ml. of blood. The blood is immediately pipetted into plastic tubes containing either normal saline or the agglutinating agent dissolved in saline. The tubes are then placed in a 37.5°C. water bath and the time noted. After exactly 3.5 minutes in the bath the tubes are removed and simultaneously shaken gently for 30 seconds and immediately returned to the bath. After exactly 7.5 minutes from the initial entrance into the bath the tubes are again removed and simultaneously shaken again for 30 seconds. Following the second shaking a definite predetermined amount from each tube is placed on a clean glass slide with a white blood cell pipette having a calibrated tip, covered with a clean 22x22 mm. coverslip, and rimmed with anhydrous lanolin. The amount placed on the slide is such that a thin smear extending to the periphery of the coverslip is obtained. The same pipette is used for each sample, and between samples it is washed with distilled water and acetone by use of an effective vacuum. It is important to get the blood from all tubes on the slides as rapidly as possible. The optimal time is to have all specimens beneath the coverslips by 12 minutes from the initial entrance into the water bath. This allows 4 minutes from the last shaking. As the time exceeds 4 minutes, the quantitative accuracy decreases. Thus, ample clean slides and coverslips must be strategically placed for easy manipulation, and the rimming of the coverslips deferred until all are in place. Four tubes can be handled easily in this time interval and with practice it is possible to transfer blood from 6 tubes in 4 minutes.

The wet smears are allowed to stand for 15 minutes and then examined by phase contrast microscopy, the control sample being examined first. With a 43x dark, medium phase contrast objective, 500 consecutive high power fields are examined and the number of leukocyte aggregates noted. The number of aggregates in 500 high power fields is termed the agglutination index and represents the degree of leukocyte agglutination in that specimen of blood. By using the specimen which is added to saline as the internal control, changes in the degree of agglutination in samples containing the agglutinating agent can be evaluated. A white blood cell count is usually done on the original blood sample. The optimal cell concentration for quantitating leukocyte agglutination is between

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5 and 10,000 cells/cu. mm. Because time is a critical factor in the dynamics of leukocyte agglutination, it is mechanically impossible to run the samples in duplicate for most experimental designs. Therefore, for a particular experimental design it is necessary to acquire enough data for statistical evaluation.

**Materials and experimental design used to evaluate the technic.** The data were obtained from observations made on blood from guinea pigs. The agglutinating agents used were a polysaccharide and two unrelated proteins. Blood was obtained by cardiac puncture and was added to normal saline or a saline solution of the agglutinating agent in the proportion of 9 volumes of blood to 1 volume of agglutinating agent.

Young, adult male, 400 to 500 Gm. guinea pigs were divided into an experimental and control group and were housed under identical environmental conditions. Each animal in the experimental group was sensitized to one of three antigens by a single intraperitoneal sensitizing injection. One group received 1 mg. crystalline egg albumin (Armour) in 1 ml. normal saline. A second group received 1 mg. bovine gamma globulin, fraction II (Armour), in 1 ml. normal saline. A third group received 1 ml. of a heart-brain infusion broth culture having a final gross turbidity of approximately 1 billion heat-killed, type III pneumococci per ml.* Control animals received also by the intraperitoneal route either 1 ml. normal saline or 1 ml. of sterile heart-brain infusion broth. The technic for quantitating leukocyte agglutination was carried out on each experimental or control blood 6 to 8 weeks after the single intraperitoneal injection. Blood from each sensitive animal was exposed to the specific antigen to which the animal had been sensitized. In the case of animals receiving pneumococci, pneumococcal type-specific polysaccharide (SIII) was used as antigen.† Blood from control animals which received a single saline injection was exposed to either egg albumin or gamma globulin. Blood from control animals receiving the sterile heart-brain infusion broth was exposed to SIII.

**RESULTS**

**Evaluation of leukocyte agglutination.** After incubation of the control specimen of blood with saline, the platelets are found to be agglutinated into aggregates of various sizes. The leukocytes are distributed around the periphery of the platelet aggregates in a uniform arrangement (fig. 1), or are dispersed between the aggregates in a random manner. When substances which promote leukocyte agglutination are added to the blood, the leukocytes tend to aggregate into small groups, either between or in association with the platelet aggregates (fig. 2). By counting the number of these leukocyte aggregates in 500 high power fields it is possible to quantitate the degree of agglutination. The number of such leukocyte aggregates in the control specimen of blood varies considerably from experiment to experiment and is dependent on leukocyte concentration and upon poorly understood factors influencing individual variation from animal to animal. However, since the control represents an internal standard for each experiment, a relative increase in agglutination in the treated blood can be quantitated. By arbitrary standards a group of at least four contiguous leukocytes was considered an aggregate. If less than four cells were grouped, or if the cells were not contiguous, they were not considered agglutinated. Figure 3 shows two groups of contiguous cells (arrows A and B) and one group of

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†The type specific pneumococcal polysaccharide was generously supplied by Dr. Michael Heidelberger.
noncontiguous cells (arrow C). In such a field two aggregates would be recorded. When the leukocyte aggregates lie between the platelet aggregates there is no problem of evaluation. However, when the agglutinated cells are associated with platelet aggregates, there is room for individual interpretation. The difficulty involves deciding whether a group of leukocytes attached to the platelets represents an agglutinated unit or whether the grouping of cells merely represents a random distribution. Because of this, consistency in interpretation from sample to sample is essential. Figures 4 and 5 are examples which were not counted as leukocyte aggregates because they resemble too closely the uniform random distribution of leukocytes around the platelet aggregates. In all cases, if there was a question of whether a grouping of cells represented agglutination or was merely an example of the random distribution, it was not counted as a leukocyte aggregate.

The dynamics of leukocyte agglutination. Figure 6 shows that time is a critical factor in evaluating leukocyte agglutination. The curve is a composite of five experiments in which samples were taken at various intervals from a specimen of blood exposed to a constant antigen concentration. The optimal time for quantitating the degree of agglutination in the system studied occurs at 10 minutes after adding the agglutinating agent. Before this time, the maximum number of leukocyte aggregates has not formed. With time the small leukocyte and platelet aggregates conglomerate to form larger
but fewer aggregates. After a critical time interval, as this takes place, it becomes impossible to quantitate the degree of agglutination. The data in this report were obtained by using a seven-minute incubation time; however, the optimal incubation time may be different for different systems.

The concentration of the agglutinating agent is also critical. Figure 7 shows the typical response of leukocytes from sensitive animals when exposed to various concentrations of specific antigen. Thus, a concentration of the agglutinating substance which falls on the peak or the ascending part of the curve must be used, since with higher concentrations conglomeration of smaller aggregates again results with decreasing accuracy of quantitation. After some experience one can recognize when excessive aggregation has taken place, since there are fewer cells in comparison to the control and only a few small aggregates with an occasional large aggregate.

The dynamic role played by the platelets in quantitating the leukocyte agglutination is difficult and somewhat precarious to interpret from the static wet smears. The author has not been successful in quantitating platelet agglutination when antigen is added to blood from sensitive animals. However, the presence of platelet aggregates is critical since the small leukocyte aggregates tend to stick to them. This reduces the formation of large leukocyte masses and allows quantitation of the leukocyte agglutination.

Fig. 2.—Leukocyte aggregate attached to agglutinated platelets. (Phase contrast x1092.)
Reproducibility and statistical evaluation of data obtained with the technic.

To determine the reproducibility of the results obtainable with the technic, blood from sensitive animals was added to saline and to quadruplicate samples of equal antigen concentration. To reduce subjective interpreta-

Fig. 3.—Groups of contiguous (A and B) and noncontiguous (C) leukocytes. (Phase contrast x1092.)

Figs. 4. and 5.—Examples of variation in random distribution of contiguous leukocytes with associated platelet aggregate. (Phase contrast x63.)
tion to a minimum, a mechanical counter was used in counting the number of aggregates in the 500 high power fields, thus eliminating knowledge of how the count was progressing as the fields were examined. Table 1 shows the agreement of quadruplicate samples for 15 experiments. Animals known to have a high degree of nonspecific agglutination in the controls were purposely selected, since there tends to be less agreement among samples when the agglutination index is high. It can be seen that there is reasonable agreement among samples except in egg albumin experiment 5 and SIII experiment 5. Egg albumin experiment 5 demonstrates that reproducibility is poor when the agglutination index is very high, and sample 4 of the SIII experiment 5 suggests that incubation time is the critical factor in this instance, since this sample was the last placed on the slide in this experiment.

Fig. 6.—Effect of time on the number of leukocyte aggregates occurring in a sensitive system.

Fig. 7.—Effect of antigen concentration on the number of leukocyte aggregates occurring in a sensitive system.
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Table 1.—Agreement of Agglutination Indices for Quadruplicate Samples Treated Identically

<table>
<thead>
<tr>
<th>Concentration Antigen Added</th>
<th>Exper. No.</th>
<th>Sample No. 1 Saline Control</th>
<th>Sample No. 2 Antigen</th>
<th>Sample No. 3 Antigen</th>
<th>Sample No. 4 Antigen</th>
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</thead>
<tbody>
<tr>
<td>Egg Albumin</td>
<td>1</td>
<td>3</td>
<td>11</td>
<td>12</td>
<td>14</td>
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<tr>
<td>.1 or 1 µg./ml.</td>
<td>2</td>
<td>8</td>
<td>43</td>
<td>42</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17</td>
<td>64</td>
<td>58</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>25</td>
<td>44</td>
<td>37</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>35</td>
<td>115</td>
<td>131</td>
<td>92</td>
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<tr>
<td></td>
<td>1</td>
<td>18</td>
<td>39</td>
<td>39</td>
<td>40</td>
</tr>
<tr>
<td>Gamma Globulin</td>
<td>2</td>
<td>3</td>
<td>42</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>.1 or 1 µg./ml.</td>
<td>3</td>
<td>11</td>
<td>29</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3</td>
<td>34</td>
<td>29</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>28</td>
<td>56</td>
<td>52</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12</td>
<td>21</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>SIII</td>
<td>2</td>
<td>22</td>
<td>37</td>
<td>42</td>
<td>39</td>
</tr>
<tr>
<td>.1 or .5 µg./ml.</td>
<td>3</td>
<td>29</td>
<td>35</td>
<td>39</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>12</td>
<td>27</td>
<td>29</td>
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<td></td>
<td>5</td>
<td>40</td>
<td>65</td>
<td>57</td>
<td>58</td>
</tr>
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</table>

Table 2 represents results obtained from 72 animals. Here, bloods from 12 sensitive and 12 nonsensitive animals were exposed to the specific antigen. The results show the lowest concentration of agglutinating agent that produces a significant increase in leukocyte agglutination for each series. The significance was evaluated by the t test. It can be seen that the technic is sensitive enough to detect a significant increase in leukocyte agglutination at antigen concentrations of 0.1 µg./ml. in a sensitive system and to show that at least 10 µg. of protein and 1 µg. of polysaccharide per ml. of blood is required to produce a significant increase in a nonsensitive system.

Stability of the leukocyte aggregates. Once the blood is placed on the slide, it is necessary to wait 15 minutes before determining the agglutination index. Within this interval the platelet aggregates tend to dissolve to varying degrees in the vicinity of the peripherally marginated leukocytes and leukocyte aggregates. This process results in an increase in number of discrete, separated leukocyte aggregates. If a specimen is examined be-

Table 2.—Demonstration of Sensitivity of Technic
(Each value represents the average from 12 experiments)

<table>
<thead>
<tr>
<th>Antigen Added</th>
<th>Average Agglutination Index</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Sensitive series</td>
</tr>
<tr>
<td></td>
<td>saline control</td>
</tr>
<tr>
<td>egg albumin</td>
<td>10.6</td>
</tr>
<tr>
<td>gamma globulin</td>
<td>7.7</td>
</tr>
<tr>
<td>SIII</td>
<td>15.9</td>
</tr>
</tbody>
</table>

*Significantly different from saline control.
fore this interval, the index will tend to be lower. After this interval the leukocyte aggregates remain stable for at least an hour, which is adequate time to examine all specimens. There is very little disruption of leukocyte aggregates due to migration of the cells, particularly of those aggregates resulting from experimental treatment. To obviate this possible source of error, however, the internal control specimen should be counted first.

The effect of a nonwettable surface. The technic has been developed by using nonsiliconed equipment. In the author’s hands, when the surfaces are made nonwettable by standard siliconing technic, the results tend to be less reproducible. Dausset et al.10 obtained false negative results when their technic was used in a siliconed system. The irregularities with siliconing in this technic seem to be mediated through the platelets. Siliconing does not decrease the degree of nonspecific leukocyte agglutination in the control specimen and the platelet aggregates are smaller, but more numerous. When a leukocyte agglutinating agent is added which also affects the platelets, the response of the platelets is more varied and less pronounced when in contact with a nonwettable surface. This in turn seems to result in immobilization of fewer leukocytes and leukocyte aggregates since, as frequently is the case in vivo, the physical processes of leukocyte and platelet agglutination are intimately related. It has already been demonstrated that with time the smaller leukocyte aggregates tend to conglomerate into larger and fewer aggregates. The presence of platelet aggregates tends to retard this process by attracting the leukocyte aggregates. With a nonwettable surface this effect of the platelets seems to be more irregular.

DISCUSSION

If one has worked with leukocytes, he appreciates their labile tendency to agglutinate and the difficulty encountered in trying to quantitate such agglutination. Others have used in vitro leukocyte agglutination as an end point in quantitating the presence of leukoagglutinating substances in serum.1,10,11 The technic presented in this report has attempted to quantitate the degree of leukocyte agglutination produced by certain agglutinating agents, and is dependent on the fact that, when leukocytes in suspension are stimulated to agglutinate, the number of leukocyte aggregates formed is in some degree proportional to the strength of the stimulus. That this is true is demonstrated in fig. 7. The difficulty in quantitating leukocyte agglutination arises from the fact that the agglutinating process involves cells sticking together to form small aggregates which in turn conglomerate to form progressively larger aggregates. The disadvantage of the described technic lies in the critical relationship of time and concentration of agglutinating agent to the agglutinating mechanism. These variables must be rigidly controlled and, if necessary, manipulated so that observations are made during the stage when the small aggregates predominate. It is necessary to establish the optimum for these variables in each system studied. A basic pattern of seven minutes incubation at 37.5°C with two 30-second periods of shaking after 3.5 and seven minutes is given. While working with a system involving serum from an animal with inflammation, the
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Author has found it necessary to incubate for 3.5 minutes and follow with a single 30-second period of shaking. Because of these two critical variables, the technic tends to give false negative rather than false positive results, because when the stage of large leukocyte aggregates is reached the agglutination index is reduced. Thus, experience in observing how leukocytes in suspension respond to agglutinating agents is necessary in order to recognize when excessive agglutination has occurred. In spite of the disadvantages it is possible with patience and care to detect microgram quantities of agglutinating agents as evidenced by the data in this report.

There is good evidence that certain clinical cases of agranulocytosis result from in vivo leukocyte agglutination which has an immunologic etiology. Although specific antileukocytic antibodies have not been satisfactorily demonstrated or characterized in these cases, it is postulated that they may be autoantibody, isoantibody, or allergic antibody in type. If they are of the auto- or isoantibody type then the antigen-antibody combination would occur directly on or in the cell and would involve an antigenic component of the cell structure. If the antibodies are of the allergic type, as would be the case in certain drug sensitivities, the antigen-antibody combination could, but would not necessarily, occur on the cell, depending on whether the antibody against the protein-allergen complex had become attached to the cell. The data in this report show that leukocytes will agglutinate in a sensitive system when antigen is added, and also in a nonsensitive system when the concentration of the antigenic substance is increased from ten to a hundred fold. This indicates that there is possibly a leukocyte-agglutinating mechanism which is triggered by an antigen-antibody reaction or by the antigen itself when in high enough concentration. Whether this agglutinating system lies entirely within the cellular structure or takes place predominately in the plasma, and to what extent, if any, this system is involved in clinical agranulocytosis must be answered by further investigation.

SUMMARY

1. An in vitro technic for quantitating leukocyte agglutination has been described.
2. With this technic the dynamics of leukocyte agglutination and the response of leukocytes to antigenic agents in both sensitive and nonsensitive systems have been studied.
3. The relationship of this study to the problem of agranulocytosis is discussed.

SUMMARIO IN INTERLINGUA

1. Es describite un technica pro le studio quantitative del agglutination leucocytic in vitro.
2. Per medio de iste technica, le dynamica del agglutination leucocytic e le responsa de leucocytos a agentes antigenic in systemas tanto sensibile como etiam non-sensibile ha esseite studiate.
3. Es discutite le relation de iste studio al problema de agranulocytosis.
REFERENCES


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