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**PASSIVE SENSITIZATION OF LYMPHOCYTES AND MACROPHAGES BY ANTGEN-ANTIBODY COMPLEXES***

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There are two recognized mechanisms by which lymphoid cells can acquire antibody activity on their surface: (1) cells synthesizing antibody can display such surface activity. Mäkelä and Nossal, extending an initial observation by Reiss et al., have demonstrated that a small percentage of cells from lymphoid organs of rats immunized with *Salmonella* flagella show specific adherence of *Salmonella* to...
their surfaces. Virtually all of the adherence-positive cells were plasma cells. This form of adherence, then, appears to represent an antigen-antibody reaction between bacterial-surface antigens and cell-bound antibody formed by the plasma cell itself. (2) "Cytophilic" antibodies initially described by Boyden and Sorkin\textsuperscript{3} can passively sensitize macrophages. For example, the incubation of rabbit "cytophilic" antibody to sheep red blood cells (SRBC) with macrophages from a nonimmunized rabbit so sensitized the macrophages that even after thorough washing they showed specific adherence of SRBC to their surface.\textsuperscript{4}

The present report describes an additional method of sensitization: antigen-antibody complexes can passively sensitize lymphocytes, plasma cells, and macrophages so that these cells bind specific antigen to their surface.

Materials and Methods.—Preparation of cell suspensions: Normal guinea pigs of the Hartley strain weighing 400 gm and rabbits weighing 2 kg were used. Lymph node cells were teased from the popliteal and cervical lymph nodes into cold 1066 medium (Baltimore Biological Laboratories). Macrophages were obtained by harvesting the peritoneal exudate 4-7 days after intraperitoneal injection of 10 ml of heavy mineral oil per guinea pig or 50 ml of oil per rabbit. Polymorphonuclear leucocytes were obtained by harvesting the peritoneal exudate 8 hr after intraperitoneal injection of 10 ml of a 1% glycogen solution. Cell suspensions were passed through a fine-mesh stainless steel cloth, washed once with 1066 medium, and resuspended in tris-phosphate buffered 1066 (pH 7.4) containing 20% fetal calf serum. The cell concentration was adjusted to $10^7$ cells/ml. Red blood cells were obtained from guinea pigs and humans by venipuncture, using a syringe rinsed with heparin 10,000 units/ml. The red cells were washed four times in 1066 medium.

For experiments in which complement (C') participation was under study, all cells were washed four times and resuspended in 1066 medium or veronal-buffered saline with added Ca\textsuperscript{++}, Mg\textsuperscript{++}, and 0.5% egg albumin.\textsuperscript{5} Fresh guinea pig serum was used as the source of C'.

Bacteria: Two strains of bacteria were used, S. paratyphi B and S. adelaide, which do not share H or O antigens. To maintain maximum motility of bacteria, liquid cultures were inoculated with bacteria taken from the edge of the growth zone on semisolid nutrient gelatin agar plates. Fresh 4- to 6-hr cultures in Antibiotic Assay Medium no. 3 (Difco) were used for adherence tests.

Antigens: Bacterial flagella and flagellin were prepared from S. paratyphi B by the methods described by Ada et al.\textsuperscript{6}

Antisera: Guinea pigs were injected intravenously with 100 $\mu$g of S. paratyphi B flagella. The animals were bled 5 days after immunization to obtain 19S antibody and after 2 weeks to obtain "primary 7S" antibody. After 4 weeks, animals were reinjected with 100 $\mu$g of flagella, and they were bled 1 week later to obtain "secondary 7S" antiserum. The immobilization titer of 5-day 19S antiserum was 1:1,000, and antibody activity was lost after treatment with 0.1 M 2-mercaptoethanol (2-ME). Primary and secondary 7S antisera had titers of 1:2,500 and 1:32,000, respectively, and antibody activity was not significantly reduced after 2-ME treatment. To obtain cytophilic antibody, guinea pigs were immunized intramuscularly with 100 $\mu$g of flagella incorporated into 0.2 ml of complete Freund's adjuvant and were bled 2 months after immunization. 7S rabbit antiserum was obtained from a rabbit injected with 200 $\mu$g of flagella in the footpads twice at a 2-week interval and bled 2 weeks after the second injection. The immobilization titer was 1:8,000 and was not reduced by treatment with 2-ME. Pepsin digestion of this antiserum was carried out by the method of Nisonoff et al.\textsuperscript{7} The concentration of pepsin-digested antibody was adjusted to that of the native antiserum. Ultra centrifugal analysis of each guinea pig antiserum was performed by the method described by Edelman et al.\textsuperscript{8}

Sensitization of cells: All cells to be sensitized were washed once after harvesting and were suspended in medium 1066 at a concentration of $10^7$ cells per ml. Ten $\mu$g of flagella in 0.05 ml were incubated with 0.05-0.2 ml of antiserum at 37°C for 30-60 min. Cells and media were then added to give a final volume of 1 ml. The suspension was usually incubated for an additional 10-60 min at 37°C. The cell suspension was centrifuged at 800 rpm for 6 min (International PR-2) and the pellet was washed once with 1066 medium. For detection of adherence-positive cells, approximately 0.01 ml of cell pellet was placed on a glass slide in the middle of a vaseline-
enclosed square. Two drops of a 4- to 6-hr culture of highly motile bacteria were added and rapidly mixed with the cells using a Pasteur pipette. Excess fluid was removed and a coverslip tightly applied. Ninety seconds after addition of the bacteria, counting of the cells was begun under phase-contrast microscopy. The criterion for adherence-positive cells (APC) was the presence of two or more adherent bacteria. Occasionally, a nonsensitized lymphoid cell or, more commonly, a nonsensitized red cell showed adherence of a single bacterium. The distinction between positive and negative lymphoid cells was usually clear, although cell clumping and the presence of aggregated complexes in the media complicated quantification. In contrast, red-cell sensitization was difficult to assess, because sensitized cells frequently had only two adherent bacteria, and vigorous movement and change in the shape of the cell made it difficult to focus simultaneously on both bacteria. To minimize subjectivity, the observer did not know the prior treatment of the cell suspensions presented to him in random sequence. The number of cells counted was 50–150 for macrophages and 100–300 for small lymphocytes and red cells. Sensitization was also obtained with 0.1 ml of a precipitate formed when 100 μg flagellin and 1 ml of guinea pig secondary 7S antisem were mixed, precipitated, well washed, resuspended in 1 ml saline, and shaken vigorously for 30 sec immediately before addition to the cell suspension. Macrophages were sensitized to SRBC by mixing the cells with 0.01 ml of washed SRBC and 0.01 ml of rabbit antisem to SRBC (previously heated to 56°C for 30 min). Adherence of the SRBC was detectable immediately under phase-contrast microscopy.

Results.—Sensitization of lymphocytes and macrophages: Table 1 summarizes the results of several individual passive sensitization experiments with guinea pig antibodies and guinea pig cells. Three of the four antisera used contained no detectable cytophilic antibody; only one antisem, from a guinea pig in which complete Freund's adjuvant had been used in the immunizing injection, contained antibody capable of sensitizing macrophages, but not lymphocytes. After mixture of antigen with the antisera, however, all the combined preparations were able to sensitize both lymphocytes and macrophages. Under the sensitization conditions employed in which virtually all macrophages became sensitized, a maximum of 18 per cent of lymphocytes became sensitized. Sensitization of both cell types was not diminished by washing three times in 1066 medium (cells were centrifuged at 800 rpm for 6 min after each washing). When the concentration of flagella was re-

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Passive Sensitization</th>
<th>Other treatment</th>
<th>Percentage of APC's</th>
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<tr>
<td></td>
<td>Antiserum</td>
<td></td>
<td>Lymphocytes</td>
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<tr>
<td>O</td>
<td>Primary 19S</td>
<td>After sensitization, washed and incubated for 2 hr at 37°C</td>
<td>N.D.</td>
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<tr>
<td>10 μg Flagella</td>
<td>Primary 19S</td>
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<td>3</td>
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<tr>
<td></td>
<td>Primary 7S</td>
<td></td>
<td>10</td>
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<tr>
<td>10 μg Flagella</td>
<td>Secondary 7S</td>
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<td>8–15</td>
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<td>Cytophilic</td>
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<td>O</td>
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<td>N.D.</td>
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<tr>
<td>10 μg Flagella</td>
<td>Secondary 7S</td>
<td>Cells suspended in 0.2% trypsin and washed 3 times before sensitization</td>
<td>N.D.</td>
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<tr>
<td>O</td>
<td>Cytophilic</td>
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<td>0</td>
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<tr>
<td>10 μg Flagella</td>
<td>Primary 7S</td>
<td>0.1 M 2-ME treatment of antiserum for 30 min at 37°C</td>
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<td>O</td>
<td>Cytophilic</td>
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N.D. = not done.
* Control showed 25% APC.
duced to 0.1 µg/ml, only 25–40 per cent of the macrophages and 5–10 per cent of the lymphocytes were sensitized, and sensitized cells had fewer bacteria on their surfaces. Both cell types were sensitized by washed specific precipitates.

Primary 19S, primary 7S, and secondary 7S antisera were fractionated by sedimentation in a sucrose gradient and the fractions were tested for sensitizing capacity. With all 7S antisera, fractions from the top but not from the bottom of the gradient had sensitizing capacity when used as specific complexes; in the case of 19S antiserum, none of the fractions had sensitizing capacity as specific complexes, presumably because of the low concentration of antibody (1:10 immobilization titer) in the fractions from the bottom of the gradient. The ultracentrifugal studies therefore confirmed the capacity of 7S antibody complexes to sensitize but gave no further information on the capacity of 19S antibody complexes to sensitize.

Rabbit macrophages were not sensitized by complexes formed from antigen and pepsin-digested rabbit antibody, even under conditions in which 98 per cent of the cells were sensitized by complexes formed with a similar amount of the native antiserum. This suggests that the heavy chains of antibody gamma-globulin must be present for antigen-antibody complexes to sensitize macrophages.

Figures 1A and B illustrate specific binding of bacteria by representative cells sensitized by antigen-antibody complexes. Figure 1C demonstrates sensitization of macrophages by SRBC-antibody complexes.

There are certain differences between antigen-antibody sensitization of lymphocytes and of macrophages. (1) The mixing of flagella, antiserum, and cells, followed immediately by twofold dilution in cold medium and washing at 4°C, resulted in sensitization of all macrophages but only a few lymphocytes. The percentage of sensitized lymphocytes increased after longer periods of incubation, during which complexes continue to aggregate. (2) Incubation of macrophages that had previously been sensitized by antigen-antibody complexes at 37°C for 2 hr resulted in the complete desensitization of the cells, whether or not C' was present. Desensitization was caused in part by phagocytosis of the surface-adherent complexes. This was demonstrated by reacting the sensitized cells with bacteria, then washing and incubating them. Following incubation, bacteria were demonstrated (by Wright's staining) within the cytoplasm of the cells. In the case of lymphocytes, partial desensitization occurred (see Table 1), but not if C' was added at the time of sensitization. This partial desensitization was presumably caused by dissociation of complexes from the lymphocyte surface. (3) Incubation of small lymphocytes with 0.2 per cent trypsin (37°C, pH 7.4) for 5 min prevented their subsequent sensitization. In the case of macrophages, however, trypsin treatment for 60 min did not prevent sensitization by complexes or by cytophilic antibody. The result with cytophilic antibody is contrary to that reported by Boyden9 for rabbit cytophilic antibody using rabbit spleen cells. (4) 2-Mercaptoethanol treatment of the antiserum, before the addition of antigen, virtually abolished the capacity of 7S (as well as 19S) antiserum to sensitize lymphocytes. Sensitization of macrophages, however, was not affected. 2-Mercaptoethanol did not noticeably affect the sensitizing capacity of cytophilic antibody.

Role of C' in sensitization: The role of C' in sensitization by antigen-antibody complexes was investigated to determine whether this mechanism of sensitization is similar to that of immune adherence (IA). IA is the binding of an antigen-anti-
Fig. 1.—Passive sensitization of macrophages and lymphocytes. Phase contrast photomicrographs × 511. (A) Two macrophages with many adherent bacteria on their surfaces. (B) Small lymphocyte with three adherent bacteria. (C) Macrophages with adherent sheep red blood cells.

body-C' aggregate to primate red blood cells or to platelets of nonprimate vertebrates.10-12 It has been reported that IA does not occur with guinea pig lymphocytes11 and is inhibited by ethylenediaminetetraacetic acid tetrasodium salt (EDTA) and other measures that exclude the participation of C".5, 11, 12

A representative experiment is illustrated in Table 2. In addition to guinea pig macrophages and lymphocytes, guinea pig red cells and human red cells were used.
Lymphocytes and human red cells were mixed before sensitization to ensure that C' had been removed from the lymphocyte suspension by the washing procedure employed. As can be seen in Table 2, the use of heated antiserum (56°C for 30 min) containing 0.02 M EDTA considerably reduced but did not abolish lymphocyte sensitization. It did not noticeably affect macrophage sensitization, but it did prevent sensitization of human red cells. Guinea pig red cells did not become sensitized. In similar experiments in which smaller amounts of antibody were used, lymphocytes were sensitized only when C' was present.

These results suggest that C' plays an important role in the sensitization by antigen-antibody complexes of lymphocytes but not of macrophages. In confirmation of previous studies,\(^5\), \(^11\), \(^12\) IA of human red cells did not occur when C' participation in the reaction mixture was excluded. In contrast to previous findings,\(^11\) however, guinea pig lymphocytes were sensitized, as indicated by bacterial adherence, by specific complexes.

**Sensitization of other cell types:** In additional experiments, it was observed that polymorphonuclear leucocytes, lymphocytes from the blood, and plasma cells from lymph nodes (immunized to unrelated antigens) could be sensitized, but that the small round cells of the peritoneal exudate (the cellular source of macrophages for these studies) did not readily become sensitized under the conditions of the experiments.

**Discussion:**—The passive sensitization of lymphoid cells by antigen-antibody complexes is not related to the surface sensitization of antibody-forming plasma cells that follows active immunization. The sensitization by antigen-antibody complexes also differs from sensitization caused by cytophilic antibody, because in the former case (1) mixture with antigen is necessary for sensitization and (2) small lymphocytes can be sensitized.

How is sensitization of phagocytes by antigen-antibody complexes related to the known capacity of these cells to ingest such complexes?\(^13\) Phagocytosis of specific complexes is presumably preceded by their binding to the surface of the cells. Antigen-antibody sensitization of phagocytes may occur through this binding step in which the specific complexes, as employed here, have combining sites "free" to interact with antigen. This conclusion is supported by the observation that macrophages sensitized by antigen-antibody complexes become "desensitized" during incubation because the complexes that were surface-adherent are phagocytized. In contrast to the antigen-antibody complexes, the majority of antisera employed did not show detectable binding to macrophages. In addition, com-
plexes formed from antigen and pepsin-digested antibody did not sensitize macrophages.

These observations suggest the following sequence for the phagocytosis of specific complexes: (1) a binding site (or sites) on the heavy chains of antibody gamma globulin is exposed as a result of the change in antibody configuration following its combination with antigen;\(^{14,16}\) (2) the gamma-globulin binding site then interacts with a receptor on the macrophage surface; (3) the complex is phagocytized. In this way, the macrophage can distinguish "free" antibody from antibody that is bound to antigen and can preferentially phagocytize the complex. Cytophilic antibody may be those gamma-globulin molecules that have a partly exposed binding site for macrophages without prior complexing to antigen. The macrophage may have other means for ingesting specific complexes that lack this binding site (or sites) such as antigen-pepsin-digested antibody complexes.

Sensitization of lymphocytes by complexes appears virtually dependent on C', unlike the sensitization of macrophages. The fact that antigen-7S antibody complexes are unable to sensitize lymphocytes after treatment of the 7S antiserum with 2-ME probably reflects the inability of such complexes to fix C'.\(^{16}\) These findings suggest that the mechanism of binding between specific complexes and lymphocytes may be different from that proposed for macrophages. The effect of C' may be caused by its capacity to increase the aggregation of antigen-antibody complexes\(^{17-19}\) or to act as a binding agent between the cell surface and the complex, as appears to be the case in IA.\(^{11}\) Until further information is available on the nature of C' participation in specific complex sensitization, conclusions on its relation to IA cannot be drawn.

The nature of the binding sites on the cell and immune complexes is also not known. The refractoriness of guinea pig red blood cells and small round cells from peritoneal exudates to sensitization by complexes may be only relative, and it is possible that it can be overcome by changing the conditions of sensitization. On the other hand, these cells may lack the appropriate surface receptor (or receptors). In contrast to macrophages, treatment of small lymphocytes with 0.2 per cent trypsin for 5 min prevents sensitization, suggesting that the receptor site either is itself protein or is bound to the cell surface by protein. Inhibition studies of passive sensitization of lymphocytes using simple sugars\(^{20}\) suggest that a polysaccharide may also be involved in the interaction between antigen-antibody-C' and cells.

Passive sensitization by antigen-antibody complexes introduces a new complication in the interpretation of immunocytological observations. Whenever antibody is associated with cells under conditions in which extracellular antigen is also present, passive sensitization by antigen-antibody complexes should be excluded before it is concluded that the antibody was formed by the cells themselves.

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3 Boydén, S. V., and E. Sorkin, Immunology, 3, 72 (1960).
THEORY OF THE FLOW OF ACTION CURRENTS IN ISOLATED MYELINATED NERVE FIBERS, VIII*

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THE ROCKEFELLER UNIVERSITY

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We continue the presentation of the theory of the isolated nerve fiber.†

Technique.—An important technical problem is to ascertain whether or not, when an anesthetic is introduced into one of the Ringer pools, longitudinal diffusion of the anesthetic along the segments of fiber in the vaseline gaps introduces a cause of error in the interpretation of experimental results. No observation has ever been made indicating that longitudinal diffusion of the anesthetic plays a significant role. A drastic example of the low rate of action of 20 mM xylocaine introduced in the distal pool upon the active zone located in the second vaseline gap was presented in reference 1c, Figure 2, 15–24 and similar results have been obtained in a number of similar experiments. Consequently, since to reach the active patch of internode the anesthetic had to diffuse through distances measured by small numbers of μ, if longitudinal diffusion of the anesthetic had played a role, this role was small in relation to that of the secondary, depolarizing action. The prevailing situation is not difficult to understand. In delaying or preventing longitudinal diffusion of the anesthetic, two mechanisms are operative. In the first place, the connective tissue surrounding the isolated fiber and the isolated fiber itself are diffusion sinks which, by binding xylocaine, decrease the concentration of the anesthetic in the diffusion front. Consequently, diffusion of the anesthetic along the isolated fiber is much slower than free diffusion in water. The second factor is this. As is often the case with weak organic bases, the free base of xylocaine is soluble in oil. For example, if a 20-mM solution of xylocaine in Ringer’s is shaken with mineral oil, so much xylocaine base passes into the oil that the oil becomes an anesthetizing agent, even for nerve with intact sheath. Consequently, an important part of the xylocaine base which is carried by longitudinal diffusion must be lost to the vaseline surrounding the isolated fiber. This process must be expected markedly to reduce the anesthetic power of the diffusing anesthetic because the anesthetic potency of the xylocainium ion is almost negligible in relation to that of the base. Under conditions such as these, it is clear that even in experiments of long duration the effect of longitudinal diffusion of the anesthetic may be entirely neglected and only the primary and secondary actions of the anesthetic (ref. 1c) need be taken into account.