Immunoprecipitation of Diffusing Antigens from Single Agar Gel-Suspended Erythrocytes (Cytoimmunodiffusion)* (34104)

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Gitlin, Sasaki, and Vuopio recently reported an immunochemical method for the quantitative determination of specific soluble proteins in individual erythrocytes (1). Independently we have developed a technique suitable for similar and related studies on membrane-limited biological particles suspended in agar and have used red cells as the most convenient model with which to explore the possibilities of the method. When diffusely suspended in an appropriate agar gel, particles are actually contained in a hole in the agar, isolated from other particles but accessible to the action of substances diffusing through the gel. Thus, AGS human red cells can be lysed by different kinds of lysins diffusing through the agar (2). The induced breakdown of the membrane converts the hole occupied by the particle into a source for the spherical diffusion of the soluble substances within the particle. These diffusing substances can be fixed in the gel around the particle by the action of different precipitating agents. The use of the precipitating action of specific antibodies permits the fixation of a given antigen in form of a granular precipitate while the rest of the soluble substances disappears by diffusion. These precipitates in agar are clearly referable to the particle from which they proceed and are readily observable under light and electron microscopy. This paper reports the methods of preparation and observations of immunoprecipitates from human red cells.

The technique consists of (1) the suspension of red cells in a thin sheet of agar; (2) diffusion of antibody through the gel surrounding the cells; (3) lysis of the cells; (4) light or electron microscopic examination of the immunoprecipitate formed as the cell contents diffuse into the surrounding agar.

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1. The thin film of AGS red cells is prepared by suspending 3× washed red cells in 1% agar in veronal-buffered saline at pH 7.4, melted and cooled to 38–40°. The mixture is allowed to gel into a thin sheet between a slide and cover glass previously prepared in the following way: a clean 50 × 75-mm glass slide is coated with two parallel strips of transparent tape. 0.025–0.050 mm thick (Scotch Brand, type 600) separated by about 20 mm; a no. 2, 25 × 25-mm coverslip is placed across these strips to obtain a chamber 25–50 μ thick. A few drops of the melted suspension are poured slowly near one of the borders of the chamber so that the fluid penetrates by capillarity into the space between slide and slip. Once the agar has gelled, the coverslip is removed and with the aid of a tubular punch 3-mm diameter discs are cut. These discs can be easily manipulated through all the operations of the procedure and the cells contained in them can be conveniently observed and counted under the microscope.

2. The diffusion of the desired antibody is obtained by transferring the discs to wells of micro concavity slides (Adams, A-1472) containing the antiserum and allowing them to remain for 30 min at room temperature. Antiserum against hemoglobin was obtained from a rabbit after 8 weekly injections of 0.5 ml of adult human red cell hemolyzate (5 mg Hb) plus 0.5 ml of complete Freund's adjuvant (Difco); tests were done on Ouchterlony immunodiffusion plates. Dr. Richard E. Tashian provided us with highly specific antisera against human carbonic anhydrases CA

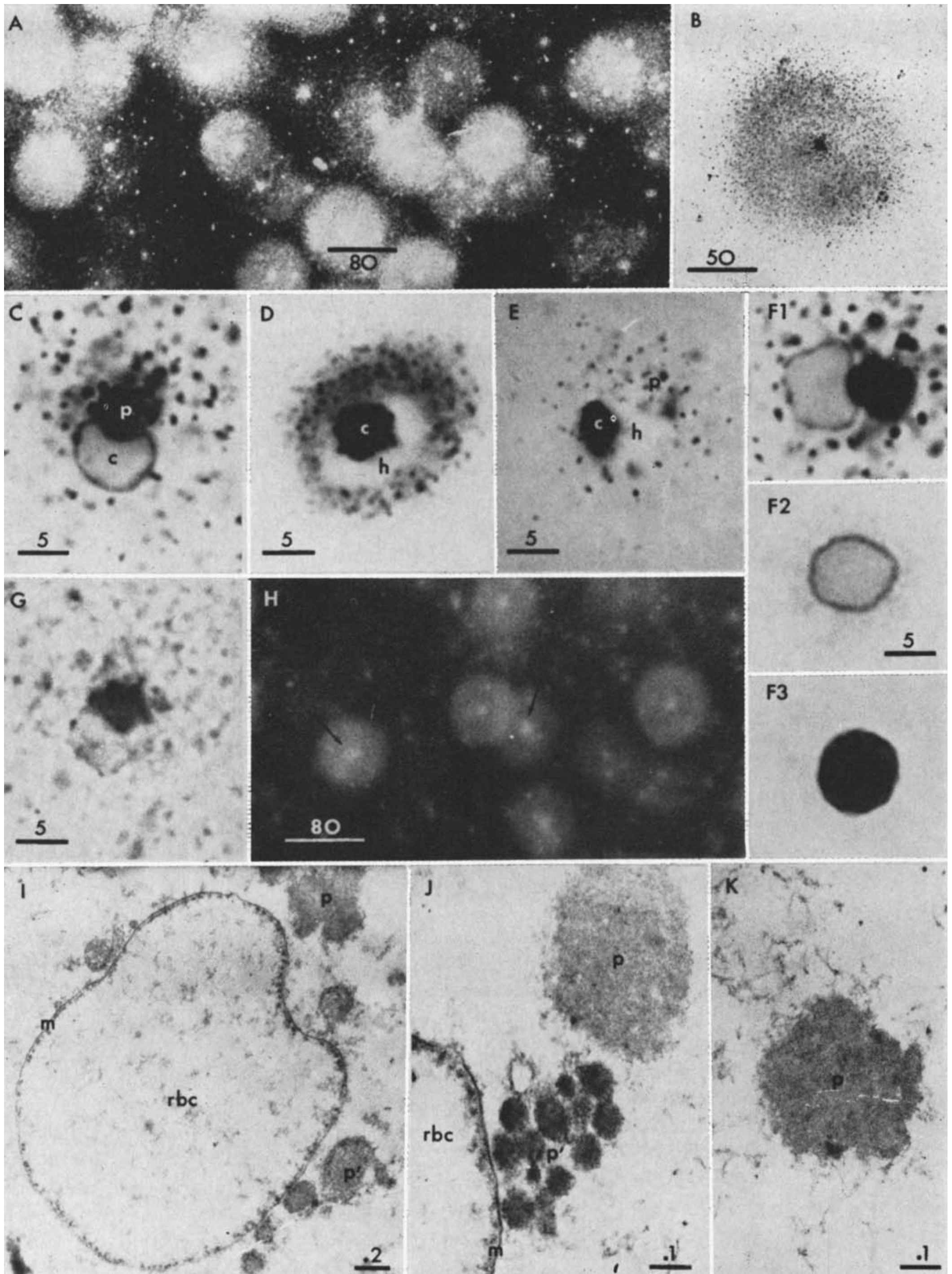

FIG. 1. A. Immunoprecipitate of hemoglobin around AGS human erythrocytes: fresh preparation observed under oblique illumination microscopy. B. Immunoprecipitate of hemoglobin around a cell; fixed, stained, and mounted preparation; the central dot is not the cell itself but a dense precipitate near the cell. C. Immunoprecipitate of hemoglobin, fixed and stained: the red cell (c)

I and CA II, prepared as described by him (3). The microslides are kept in petri dishes lined with wet filter paper to avoid evaporation.

3. The rupture of the cell membrane can be obtained either chemically by the addition of Triton X-100 (1–2%) to the incubation serum or by immunolysis. When the antibodies are in rabbit sera the addition of guinea pig complement together with the anti-human red cell lysin naturally occurring in rabbit serum produces the lysis of the cells (2). The lytic action of Triton X-100 is completed in 1 or 2 min and the immunoprecipitate around the lysed cells becomes apparent almost immediately. This process can be directly observed microscopically as described below. Immunohemolysis is, at room temperature, a slower process, taking several hours. Normally the discs are maintained at room temperature for 12 hr regardless of the way in which lysis was induced.

4. The immunoprecipitates can be examined directly with the discs in the wells or between slide and coverslip with darkfield or oblique illumination microscopy. Oblique illumination produces the brightest images (Fig. 1A). Phase-contrast microscopy also gives good images. The best conditions for observation are obtained with permanent fixed and stained preparations. The discs are washed in saline for 30 min to remove all possible serum proteins, fixed in osmium tetroxide (4) for 30 min, washed in distilled water 5 min, and stained with Heidenhain's iron hematoxylin (5) according to the following schedule: mordanted 15 min in 4% ferric ammonium sulfate, washed 2× for 15 min each in distilled water and transferred to 5% hematoxylin solution until the precipitates become dark. There is no need for differentiation. Washing in alkaline water turns the stained precipitates deep blue. Discs are mounted without dehydration in modified (6) Apathy's gum syrup for permanent preparations.

These preparations clearly show the lysed cells and the areas of distribution of the immunoprecipitates around them. The precipitate is formed of small, well defined granules (Fig. 1B). Under suitable magnification (Fig. 1C) it is possible to identify the membrane of the lysed red cell (c). When the erythrocyte has been destroyed by immunolysis the membrane retains its size and shape. The dense clumps of granules (p) routinely observed just outside the cell and the distribution of granules around this clump suggests that with immunolysis the release of the cell contents occurs through a single point (Fig. 1C). When lysis is produced by Triton X-100 the single large extracellular spot does not appear (Fig. 1 D, E); the membrane usually retracts and ap-

has been lysed by immunolysis and appears empty; it retains its shape and size (F1 is a similar image); a large precipitate (p) appears close to the membrane and many other smaller particles surround the cell. D. Immunoprecipitate of human carbonic anhydrase I; the cell (c) has been lysed with Triton X-100 and appears shrunk inside the hole (h) originally produced in the agar by the intact cell; the zone of precipitates (p) appears around. E. Immunoprecipitate of human carbonic anhydrase II. F. Comparative images from fixed and stained preparations of: 3, an AGS intact erythrocyte, unlysed; 2, an AGS erythrocyte lysed by control rabbit serum containing no specific antibodies, and 1, an AGS erythrocyte lysed by rabbit serum containing antibodies to human Hb. G. Peroxidase-positive reaction of the hemoglobin immunoprecipitates; compare with C: the granules are clearly stained in brown, but the cell membrane is not. H. Immunoprecipitate of hemoglobin obtained with rabbit antibodies; the preparation has been treated with fluorescent anti-rabbit gamma globulin antibody: the precipitates appear fluorescent: the arrows point to nonfluorescent intracellular spaces. The cell membranes appear to fluoresce. I. Electronmicrograph of an immunolysed AGS erythrocyte (rbc) showing a well defined membrane (m) with granular formations on the inner surface and immunoprecipitates (p and p′) surrounding the cell. J. Higher magnification showing part of a cell membrane (m), fine-grained precipitate particle (p), and coarse-grained particles (p′). K. High magnification of fine-grained precipitate particle (p), found at some distance from the cell, and its relationship with the fine fibrillar precipitate. In each illustration the scale is shown in micra.

pears as a clump (c) inside of the hole (h) produced in the agar by the shrinkage of the cell. Cells not lysed and cells lysed but without precipitates around them can also be identified at higher magnifications (Fig. 1F-1, 2, 3). When there is only a small amount of antigen present, as, for instance, in the case of carbonic anhydrase II (CA II), high magnification of the fixed stained material permits the identification of the immunoprecipitate granules (Fig. 1E).

The granular precipitates surrounding AGS-human red cells treated with rabbit anti-human Hb serum were shown to contain rabbit gamma globulin by treating them, after exhaustive washing, with fluorescein-labeled goat anti-rabbit gamma globulin. The immunoprecipitates showed strong fluorescence (Fig. 1H). Similarly, the presence of hemoglobin in these precipitates is indicated by their peroxidase activity. By applying the benzidine reaction (7), the precipitates, but not the cell membrane, take a brown color (Fig. 1G).

The good preservation of the cells and the precipitates obtained by this technique and their fixation with osmium tetroxide permits observation and study under the electron microscope. In this case a thicker film of agar (0.5 mm) is prepared; the discs obtained are treated in the same way until after fixation. They are then dehydrated, embedded in Araldite 502 according to Luft (8) and sectioned. Intact human erythrocytes suspended in agar show no difference under electron microscopy from those observed in sections of tissues. The agar surrounding unlysed cells is completely free of visible structure. The immunolysed cells are visible as irregular formations, constituted by a well defined membrane (m) covered inside by granular formations (Fig. 1 I). These granules are the same diameter (approximately 100–150Å) as the membrane-associated particles described by Weinstein and co-workers (9, 10). When antibodies against diffusible substances have been used, precipitates are visible in the agar (Fig. 1 I, J, K,) as fine fibrillar formations and granules of different sizes, some of fine-grained (p) an others of coarse-grained ultrastructure (p′).

Summary. When agar gel-suspended erythrocytes are exposed to a specific antibody and then lysed, the intracellular soluble antigen diffuses in all directions and reacts with the surrounding antibody. This reaction produces a characteristic granular precipitate around individual cells which, upon proper fixation, can be studied by light or electron microscopy.

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