Cournand, A., *et al., Am. J. Physiol.,* 1948, v153, 309.

4. Kramer, K., Thurau, K., Deetjen, P., *Pflüger's Arch.,* 1960, v270, 251.

5. Mériel, P., Galinier, F., Suc, J. M., *Proc. 1st Internat. Congress of Nephrology,* Karger & Basle, New York, 1961, p224.

6. Munck, O., *Renal circulation in acute renal failure,* C. C Thomas, Springfield, Ill., 1958.

7. Oedman, P., *Acta radiol.,* 1956, v45, 1.

8. Smith, H. W., *The kidney in health and disease,* Oxford Univ. Press, New York, 1950.

9. Sparling, C. M., *Recording and quantitative interpretation of dye dilution curves obtained by reflectometry in red or infrared light.* Van Gorcum & Co., Groningen, 1961.

10. Stewart, G. N., *Am. J. Physiol.,* 1921, v57, 27.

11. Warren, J. V., Brannon, E. S., Merrill, A. J., *Science,* 1944, v100, 108.

Received October 8, 1962. P.S.E.B.M., 1962, v111.

Passive Acute Glomerulonephritis Induced by Antigen-Antibody Complexes Solubilized in Hapten Excess.* (27915)

ROBERT T. MCCLUSKEY, BARUJ BENACERRAF AND FREDERICK MILLER
Department of Pathology, New York University School of Medicine, New York City

The studies of Germuth(1) and of Dixon *et al.*(2) have established that the glomerular lesions of serum sickness occur during the phase of immune elimination of the antigen, when antigen-antibody complexes circulate in the blood, and that antigen can be detected in the lesions by the fluorescent antibody technic. Recent experiments from this laboratory have shown that the lesions of serum sickness, especially acute glomerulonephritis, can be induced passively in mice by intravenous injection of adequate amounts of antigen-antibody complexes prepared from rabbit(3) or mouse antibody(4) and solubilized in antigen excess. The fate and distribution of the complexes have been studied by the fluorescent antibody technic(5). These findings have been confirmed and extended by Mellors and Brzosko(6), who also observed the localization of the injected complexes in the glomerular lesions by immunofluorescence. The significance of this observation was weakened, however, by their finding that normal rabbit gamma globulin conjugated with fluorescein, when injected intravenously, also localized to some extent in normal mouse glomeruli(6). The possibility that this property of the gamma globulin was the result of conjugation with fluorescein was not investigated.

To provoke glomerulonephritis by intravenous injection of soluble antigen-antibody complexes, large amounts of antigen have to be used in order to maintain the complexes soluble. While control experiments have shown that similar injections of foreign proteins do not induce the lesions of glomerulonephritis by themselves(3), it seemed desirable to modify the technic to eliminate this objection. In experiments reported here, acute glomerulonephritis has been produced in mice with rabbit anti-2,4-dinitrophenyl bovine gamma globulin (DNP BγG) antibodies precipitated at equivalence with 2,4-dinitrophenyl bovine serum albumin (DNP-BSA) and dissolved in excess hapten, ϵNH_2 DNP lysine. The rapid diffusion of the small molecular hapten allows formation of the larger immune aggregates, which have been shown by the fluorescent antibody technic to localize characteristically in the reticuloendothelial system(7) and in glomeruli(5). Control experiments carried out with equal doses of purified rabbit anti-DNP antibody alone showed absence of similar localization.

Materials and methods. Antigen. Bovine gamma globulin (BγG) and bovine serum albumin (BSA) (Armour Pharmaceutical Co., Illinois) were conjugated with 2,4-dinitrofluorobenzene as described previously(8).

* Supported by U. S. Public Health Service grant, Nat. Inst. of Allergy and Infect. Dis. and in part by Health Research Council of Dept. of Health, City of New York.

The number of DNP groups per molecule of conjugates was determined spectrophotometrically at 360 mμ using 17.400 as the molar extinction coefficient for DNP. The preparations used were heavily conjugated: DNP BγG 81 groups and DNPBSA 40 groups.

Immunization. Adult rabbits were immunized with DNP BγG. They were injected twice intramuscularly at a 2-week interval with 5 mg of the antigen in complete adjuvant (Difco Laboratories, Mich.). Then, 2 mg DNP BγG were injected intravenously twice a week for 2 weeks. The animals were bled 7 days later, the sera assayed qualitatively for antibody and the strongest sera pooled.

The pooled rabbit anti-DNP BγG serum was titrated to equivalence with DNPBSA by the quantitative precipitin technic(9). It contained 4.3 mg of anti-DNP antibody/ml, specifically precipitable with 0.4 mg of DNPBSA.

Preparation of soluble complexes. The amount of DNPBSA required to precipitate the antibody at equivalence was added to the pooled anti-DNP BγG serum. After an hour at 37°C, the precipitate was centrifuged and washed 3 times with cold saline. A solution of ϵNH_2DNP lysine, 2×10^{-3} Molar (Mann Laboratories, N. Y.) was then added to dissolve the precipitate in such a manner that the final solution contained 10 mg of antibody/ml. Complete solubilization was obtained in 2 hours at 37°C.

Preparation of purified anti-DNP antibodies. This procedure was carried out as previously described(10). After extensive dialysis, the preparation, while free from antigen, still contained some ϵNH_2DNP lysine which had been used to dissociate the antibody, because of the high avidity of these antibodies.

Fluorescent antibody technic. Sheep antiserum against rabbit gamma globulin was kindly provided by Dr. G. J. Thorbecke. This antiserum was absorbed as described previously(11) with a fraction of rabbit serum from which gamma globulin had been removed by precipitation with sodium sulfate. Following absorption, the sheep antiserum showed in Ouchterlony plates only a single line against rabbit serum, and which by immunoelectrophoresis was seen to represent anti-gamma globulin. A gamma globulin fraction of the sheep antiserum was prepared with 0.4 saturated ammonium sulfate and following dialysis was conjugated with fluorescein isothiocyanate according to the method described by Mellors(12). Nonspecific staining was removed by repeated absorptions with homogenates of mouse tissue.

For fluorescent staining, blocks of tissue were frozen in liquid nitrogen, sectioned at 2 μ in an International Harris cryostat, air dried, fixed in acetone for 10 minutes, and immersed in normal saline buffered with 0.02 M phosphate at pH 7.0. Sections were covered with conjugated sheep anti-rabbit gamma globulin for 30 minutes, and then washed 3 times with buffered saline. The specificity of the staining was shown by the failure of the conjugate to stain normal mouse tissue and by the marked suppression of staining by prior application of unconjugated sheep anti-rabbit gamma globulin for 45 minutes.

Sections were examined with a Zeiss standard microscope with an Osram HBO 200 watt mercury lamp and a dark field condensor.

For routine histology, tissues were fixed in 10% formalin and stained with hematoxylin and eosin.

Animals. Swiss Webster male mice weighing 25-30 g were used.

Results. A. *Production of acute glomerulonephritis.* Two experiments were performed. Exp. I. At 10 a.m., 2 groups of mice were injected intravenously in the tail vein with 1 mg or 3 mg of anti-DNP antibodies as complexes with DNPBSA solubilized with ϵNH_2DNP lysine. Four of the 10 mice which received 1 mg and 6 of the 11 mice which received 3 mg of antibody died of anaphylaxis. The surviving mice were injected again with the same doses at 5 p.m. and at 9 a.m. the next morning. No deaths were observed after the second or third injection. Both groups of mice were sacrificed 24 hr after the last injection. Histologic examination showed that of the 6 mice which

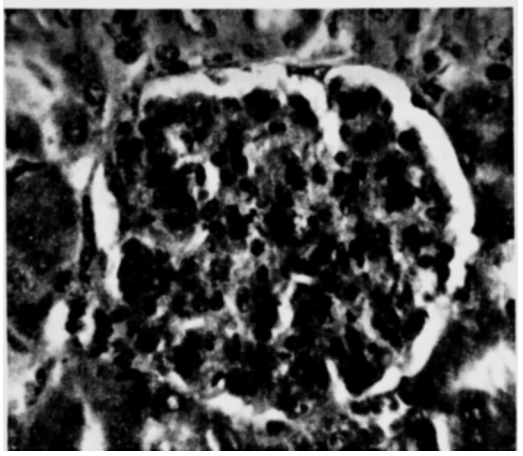

FIG. 1. Glomerulus from mouse given 3 injections of soluble complexes in 24 hr. Glomerulus shows swelling of cells, endothelial proliferation and contains a few neutrophiles. Hematoxylin and eosin. X250.

received 3 × 1 mg of anti-DNP as complexes 4 showed mild, focal glomerulitis, characterized by swelling of endothelial cells, and increased cellularity. Of the 5 mice which received 3 × 3 mg of antibody as complexes, 4 had well-developed acute glomerulonephritis with swelling of glomerular cells, hypercellularity and neutrophile infiltration (Fig. 1).

Exp. II. A third group of mice was injected with complexes containing 1 mg of antibody. The 5 mice which survived the anaphylactic reactions were reinjected at the times specified with soluble complexes containing the following amounts of antibody: one hour, 2 mg; 7 hours, 3 mg; and 24 hours, 3 mg. When sacrificed 24 hours later, all these mice showed on histologic examination well-developed lesions of acute glomerulonephritis. It is clear that more severe lesions (9 positive out of 10 mice) were obtained with the larger amounts of antibody. This was the same dosage which had been found to be effective with the rabbit anti-ovalbumin system(3).

B. *Fluorescent antibody studies.* A first group of 6 mice received intravenously immune complexes dissolved in hapten excess containing 3 mg of anti-DNP antibodies; 2 mice died of anaphylaxis after 15 minutes, 2 mice were killed 3 hours after injection, and 2 mice were sacrificed after 24 hours.

A second group of 3 mice received 3 doses of the complexes in 24 hours, each containing 3 mg of anti-DNP antibody. They were sacrificed 24 hours after the last dose. As controls, a third group of 3 mice received intravenously 3 mg of purified rabbit anti-DNP antibodies and were sacrificed at 3 hours and a fourth group received 3 intravenous injections in 24 hours of 3 mg of the same purified antibodies and were killed 24 hours after the last dose. These control mice did not receive antigen. The lungs, liver, spleen, and kidneys were examined for rabbit gamma globulin by the fluorescent antibody technic.

In the animals which died of anaphylaxis 15 minutes after an injection of immune complexes, specific fluorescence appeared to line the sinusoids of liver and spleen; some specific fluorescence was seen in a few Kupffer cells but none in the kidneys. In mice killed 3 hours after injection of the complexes, many Kupffer cells and macrophages in the red pulp of the spleen stained heavily for rabbit gamma globulin (Fig. 2); in the kidneys, specific fluorescence, which seemed to be within cells, was observed focally in a few glomeruli. In the mice killed 24 hours after a single injection, only a very few Kupffer cells and splenic macrophages showed specific fluorescence; the staining was quite faint. In animals which received 3 doses of complexes and were killed 24 hours later, faint fluorescence was seen in a very few Kupffer cells and in a few splenic macrophages; all glomeruli stained heavily for rabbit gamma globulin (Fig. 3). The kidneys of the mice injected with anti-DNP antibody alone showed complete lack of specific fluorescence. Only a few Kupffer cells and splenic macrophages stained very faintly in the mice sacrificed 3 hours after a single injection. None of the other organs stained for rabbit gamma globulin.

Discussion. Acute glomerulonephritis can be induced passively in mice by intravenous injection of antigen-antibody complexes using a well-defined immunological system (DNP). The immune complexes can be dissolved in excess of hapten instead of excess complete antigen without affecting the severity of the glomerular lesions, showing that the excess

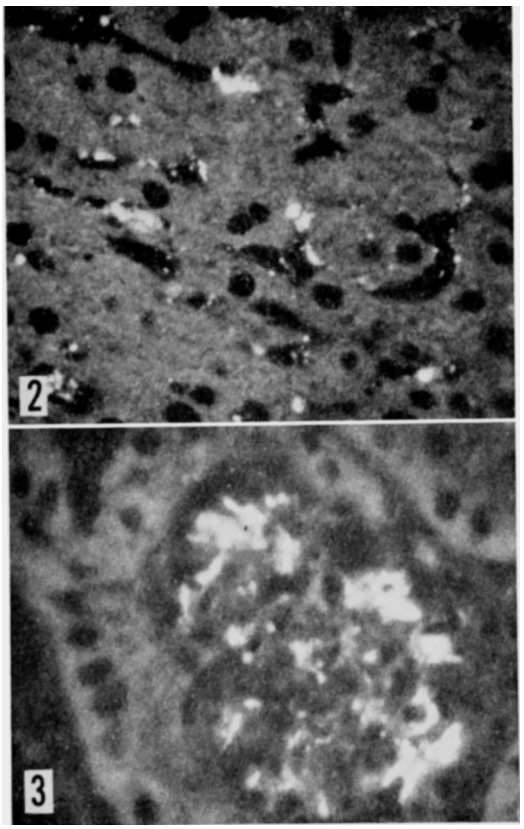

FIG. 2. Liver from mouse killed 3 hr after a single injection of soluble complexes. Many Kupffer cells show abundant specific fluorescence. Section stained with fluorescein conjugated sheep anti-rabbit γ-globulin. X240.

FIG. 3. Glomerulus from mouse killed 24 hr after 3 injections of soluble complexes showing marked accumulation of specific fluorescent material. Section stained with fluorescein conjugated sheep anti-rabbit γ-globulin. X180.

protein antigen previously(3) used in the systems (ovalbumin-anti-ovalbumin and BSA-anti-BSA) was not necessary for development of the lesions. The further use of such simple immunological systems, where the immunological specificity is well defined and energy of binding can be measured, may provide information concerning the pathogenesis of the glomerular lesions. The studies with fluorescent antibodies confirm the presence of the complexes in the glomerular lesions. The soluble immune complexes injected intravenously are cleared by the RES for the most part, as previously reported(7); after a single injection, only a small fraction can be seen to localize in the glomeruli. Their arrest in glomeruli may be due to the selective filtrating function of these structures which may cause a change in the solubility of the complexes and lead to their deposition.

The complexes phagocytized by the RES are rapidly metabolized by these cells, as shown by the observation that almost all of the gamma globulin has disappeared by 24 hours after an injection. In contrast, the complexes that are caught in the glomeruli appear not to be as subject to degradation processes, either because they are trapped within cells which are relatively incapable of metabolizing them or because they are located along the basement membrane and are inaccessible to cellular attack. With repeated injections, greater trapping of complexes occurs in glomeruli, possibly as the result of the increased stickiness of the endothelial cells caused by damage due to the initial localization.

The absence of kidney localization when equal amounts of purified anti-DNP antibodies were injected shows that localization of anti-DNP antibodies in the glomeruli depends upon formation of immune complexes.

Summary. Acute glomerulonephritis has been produced passively in normal mice by soluble antigen-antibody complexes prepared from rabbit anti-DNP BγG which was precipitated at equivalence with DNPBSA and dissolved in excess hapten, ϵNH_2DNP lysine. The fate and distribution of the injected antigen-antibody complexes and of purified rabbit antibody, which was used as a control, were studied by the fluorescent antibody technic. The complexes were rapidly and efficiently phagocytosed by the cells of the reticuloendothelial system and had largely disappeared from these cells within 24 hours. In contrast, there was only slight localization of complexes within glomeruli following a single injection, but with 3 injections in 24 hours, which resulted in production of acute glomerulonephritis, marked accumulation of complexes occurred in glomeruli; this was apparent 24 hours after the last injection. Purified rabbit antibody did not localize in glomeruli and only in trace amounts in the cells of the reticuloendothelial system.

1. Germuth, F. G., Jr., *J. Exp. Med.*, 1953, v97, 257.

2. Dixon, F. J., Vasquez, J. J., Weigle, W. O., Cochrane, C. G., *A.M.A. Arch. Path.*, 1958, v65, 18.

3. McCluskey, R. T., Benacerraf, B., Potter, J. L., Miller, F., *J. Exp. Med.*, 1960, v111, 181.

4. Miller, F., Benacerraf, B., McCluskey, R. T., Potter, J. L., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 706.

5. Cooper, N. S., McCluskey, R. T., Benacerraf, B., Miller, F., *Atti del X Congresso della Lega Internazionale contro il Reumatismo,* Minerva Med., Roma, 1962, vII, 814.

6. Mellors, R. C., Brzosko, W. J., *J. Exp. Med.*, 1962, v115, 891.

7. Benacerraf, B., Sebestyen, M., Cooper, N. S., *J. Immunol.*, 1959, v82, 131.

8. Benacerraf, B., Levine, B. B., *J. Exp. Med.*, 1962, v115, 1023.

9. Kabat, E. A., Mayer, M. M., *Experimental Immunochemistry,* 2nd ed., Charles C Thomas, Springfield, Ill., 1961.

10. Edelman, G. M., Benacerraf, B., Ovary, Z., Poulik, M. D., *Proc. Nat. Acad. Sci.*, 1961, v47, 1751.

11. Thorbecke, G. J., *J. Exp. Med.*, 1960, v112, 279.

12. Mellors, R. C., *Analytical Cytology,* 2nd ed., McGraw-Hill Book Co., New York, 1959.

Received October 9, 1962. P.S.E.B.M., 1962, v111.

Erythrocyte Survival in Guinea Pigs. (27916)

L. H. SMITH AND T. W. MCKINLEY, JR.

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.*

Using calculations based on appearance of Fe^{59}-labeled erythrocytes in peripheral blood, Everett and Yoffey(1) estimated erythrocyte life span for the guinea pig to be 83 days. From a survival curve of erythrocytes labeled *in vivo* with Cr^{51}, Grönroos(2) concluded that 65 days was a good estimate of the life span of guinea pig erythrocytes. In previous studies of erythrocyte survival of mice and rats we used the technic whereby cells are labeled *in vitro* with Cr^{51}. The present series of experiments was performed to obtain comparative data for guinea pigs and to determine survival of autologous, isologous, and homologous erythrocytes of this species.

Methods. Male and female strain 2 and 13† guinea pigs weighing 700-800 g and 2-3 months old at the beginning of each experiment were used. From a punctured ear vein, 1-2 ml of donor blood were allowed to flow freely into a glass test tube containing about 100 IU of heparin. To each ml of blood 10-20 μc of Cr^{51} (as $Na_2Cr^{51}O_4$) were added. Concentration of chromium metal ranged from 0.04-0.09 μg/ml of blood. Chromation time at room temperature was 60-90 minutes, during which period the mixture was agitated frequently by tube inversion. Forty volumes of 0.9% NaCl were added and the blood was then centrifuged. The supernatant was discarded and sufficient saline added to the labeled cells to reconstitute to original blood volume. One ml of this preparation was injected into the external jugular vein of each anesthetized (Nembutal-ether) recipient. The first blood sample (0.1 ml) was collected 24 hours later from a punctured ear vein, and the sample discharged into 1.0 ml of water. Subsequent blood samples were collected likewise, at 7-day intervals for 10 weeks. Blood for duplicate microhematocrits was also obtained at each period. Radioactivity was measured in a well-type scintillation counter and triplicate 5000-count determinations were made for each sample. Counts were corrected for background, physical decay of isotope, and hematocrit. The 24-hour count was considered 100% and values for subsequent samples were expressed as percent of the initial 24-hour activity.

To determine the proportion of labeled cells, P, on day t, we used the following

* Operated by Union Carbide Corp. for U. S. Atomic Energy Commission.

† Both strains are maintained at this laboratory by brother-sister mating. Original breeders were obtained from Nat. Inst. of Health.