19S Antibodies as Mediators of Pulmonary Edema Produced in Rats by Nephrotoxic Serum[1] (34416)

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Previous studies have shown that the pulmonary edema produced in rats by nephrotoxic serum is a complement-dependent tissue injury, apparently not involving the participation of some vasoactive substances (histamine, 5-hydroxytryptamine and kinins) or polymorphonuclear leukocytes (1). The present investigations were carried out in order to characterize the antibodies in nephrotoxic sera responsible for the production of the lung lesions. The data to be presented show that antibodies are 19S immunoglobulins with striking complement-fixing properties.

Material and Methods. Wistar rats of both sexes, weighing 120–200 g, were used. Rat kidney cortex homogenate (RKC) and rabbit anti-RKC serum (nephrotoxic serum) were obtained and used exactly as previously described (1). Rat lung homogenate (RL) was prepared as follows: the rats were anesthetized with ether, bled out by section of the large abdominal vessels, the trachea was cannulated and the thorax was opened. A polyethylene cannula was inserted into the pulmonary artery, the perfusion was started and the left ventricle was opened. The lungs were perfused (under air insufflation through a tracheal cannula) with 80–100 ml of 0.15 *M* NaCl. The lungs were removed, cut in slices, and homogenized using a Potter-Elvehjem-type homogenizer in a volume of 0.15 *M* NaCl in order to obtain a 10% tissue suspension (w/v). The homogenate was centrifuged to remove the large particles, the protein content was determined and stored at −20°. The preparation used contained 27.9 mg of protein/ml.

Complement fixation. The method employed was essentially as described by Wasserman and Levine (2). The reaction mixture contained antigen (RL) at the concentration of 0.55 μg of protein/ml, rabbit anti-RKC serum or γ-globulin fractions in the appropriate dilutions and 1.7 CH_{50} units of guinea pig complement in a total volume of 3.5 ml. The diluent used was triethanolamine-buffered saline (TBS), at pH 7.4, ionic strength 0.15, containing 1.5×10^{-4} *M* Ca^{2+}, 5×10^{-4} *M* Mg^{2+} and 0.05% gelatin. The mixtures were placed in the refrigerator at 4° for 16–18 hr, and sensitized erythrocites were then added (0.5 ml of sensitized erythrocytes at 5×10^7 cell/ml). Controls of complement alone or complement with either antigen or antibody were incubated in the same manner. After 60 min at 37°, the cells were sedimented by centrifugation and the optical density in the supernatant fluids measured at 413 mμ. The complement fixed was determined by subtracting the complement activity remaining in the test samples from the complement activity of the controls. The percentage of complement fixed was calculated as the ratio of the complement fixed to the activity of the controls.

Gel diffusion method. Precipitin analysis by double diffusion in 1% agar gel was performed as described by Ouchterlony (3).

Protein determinations. Protein concentrations were either measured by the method of Lowry *et al.* (4), or estimated by absorption at 280 mμ, assuming a value for $E^{1\%}_{1\,cm}$ of 10.

Production of the acute pulmonary edema (APE). After the intravenous injection

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through the tail vein with rabbit anti-RKS serum or with its γ-globulin fractions, the rats were observed up to a period of 60 min and the APE symptoms recorded as follows: no symptoms (—); slight dyspnea (+); severe dyspnea (++); dyspnea plus cyanosis (+++); dyspnea and cyanosis followed by death (++++). At the conclusion of each experiment, the thorax of each animal was opened and examined for the presence of edema and hemorrhage of the lungs. Eventual microscopical observations were made, using lung tissues fixed in 10% neutral formaldehyde, imbedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Results. 1. Preparation of γ-globulins. The γ-globulin fraction was obtained from pools of rabbit anti-RKC sera. It was prepared by adding 1 vol of saturated $(NH_4)_2SO_4$, pH 6.5, to 2 vol of rabbit anti-RKC serum with constant stirring. The mixture was allowed to stand for 15 min and then was centrifuged. The sediment was washed twice with 40% saturated $(NH_4)_2SO_4$, centrifuged and dissolved in 1 vol of 0.15 *M* NaCl corresponding to a half of the original volume of the serum. The globulins were dialyzed overnight at 4° against 50 vol of 0.15 *M* NaCl. Fine precipitates were removed by centrifugation and the globulins stored at —20° until use.

2. Gel filtration. Four-ml aliquots of the rabbit γ-globulin preparations containing 25–30 mg of protein/ml, were applied to a glass column (80.0 × 3.0 cm) packed under gravity flow with Sephadex G-200 (Pharmacia, Uppsala, Sweden) to height of 75 cm, and equilibrated with 0.3 *M* NaCl. Elution was accomplished at 4° at a flow rate of 16 ml /hr. Fractions were collected with an automatic device (4.0 ml each), assayed for protein content by spectrophotometry at 280 mμ and pooled according to elution patterns. The elution positions of substances with known molecular weights were determined in the same manner. For this purpose, purified hemoglobin from the snail *Biomphalaria glabrata* (kindly donated by Dr. A. G. A. Neves from the Department of Biochemistry, ICB, UFMG) with a molecular weight of 1.6×10^6 (5) was used to define the V_0 (void volume) of the column and crystalline bovine

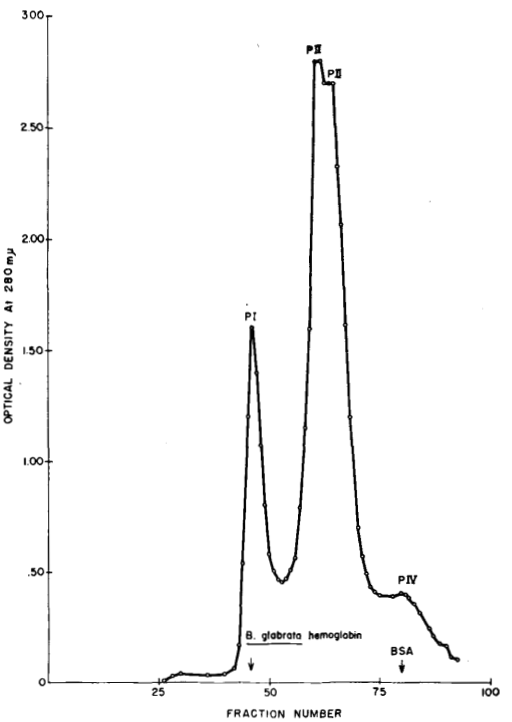

FIG. 1. Elution pattern of rabbit γ-globulins anti-RKC homogenate resulting from filtration through a Sephadex G-200 column.

serum albumin (Sigma Chem. Co.) was selected as 4*S* marker. The markers were used at a concentration of 1% and were dissolved in 0.3 *M* NaCl. The distribution of protein obtained is illustrated in Fig. 1. The central fractions corresponding to peak of protein PI (19*S* component), and to peaks PII and PIII (7*S* component) were pooled, concentrated by ultrafiltration to about 5.0 ml, dialyzed against phosphate–saline buffer, pH 7.4, ionic strength 0.15, and assayed for protein content.

3. Immunodiffusion analysis. The pattern of reaction obtained by double-diffusion gel precipitation test is exemplified in Fig. 2. As shown, rabbit serum anti-RKC homogenate and its total γ-globluins or the 7*S* component preparations, formed bands of precipitation against both RKC or RL. On the other hand, the 19*S* component failed to show any visible precipitin band.

4. Complement fixation. Rabbit anti-RKC sera and the 19*S* and 7*S* components were

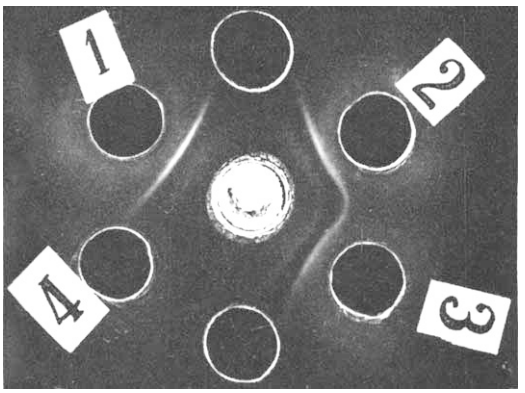

FIG. 2. Double-diffusion gel precipitation test; peripheral wells: (1) rabbit anti-RKC serum; (2) rabbit anti-RKC γ-globulin preparation; (3) 7*S* component anti-RKC; (4) 19*S* component anti-RKC; central well: rat kidney cortex (RKC) homogenate.

studied for its ability to fix complement after reaction with antigen(s) present in RL homogenate. Each preparation was studied at several different concentrations. The antigen–antibody complexes were allowed to form in the presence of complement. The results from one experiment are shown in Fig. 3. It is seen that the 19*S* component is much more effective in fixing complement than the 7*S* component. It was also found that in the majority of the rabbit anti-RKC sera studied (not represented in Fig. 3), a complement fixation of the order of 50% was obtained when sera were diluted 1:600 to 1:800.

5. APE production. Rats intravenously injected with 0.5 ml of rabbit anti-RKC serum or with its γ-globulin preparation (11.16 mg of protein for each animal), depicted symptoms of APE similar to those previously described (1). The same symptoms were also observed when the 19*S* component (1.34 mg of protein for each animal) was used. Nevertheless, no symptoms were observed in the rats injected either with normal rabbit serum (1.0 ml) or with the 7*S* component (3.65 mg of protein for each animal). Table I shows one of these experiments. The LD_{50} end point of the 19*S* component was determined by the method of Reed and Muench (6). It was about 1.22 mg of protein when rats weighing 150–170 g were used. Rats injected with large doses of some 7*S* preparations showed, sometimes, slight symptoms of dyspnea and cough which however, disappeared after 2–3 hr.

6. Reduction and alkylation of 19S component. The proteins were dialyzed against 0.2 *M* 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, N.Y.) during 18 hr and overnight against 0.22 *M* iodoacetamide (Calbiochem, Los Angeles, Ca.). The diluent used was Tris–HCl buffer, pH 8.0. Following the alkylation, the protein was dialyzed against phosphate–saline buffer, pH 7.4, ionic strength 0.15. After these treatments, the 19*S* antibodies were unable either to produce symptoms of APE or to fix complement.

7. The appearance in rabbit serum of anti-RKC antibodies involved in APE production. Normal rabbits were subcutaneously injected with RKC homogenate (45 mg of protein) emulsified in 2 vol of complete Freund adjuvant. The inoculum was equally distributed among the hind legs and over the abdominal wall. Serum samples were obtained immediately before the injection of

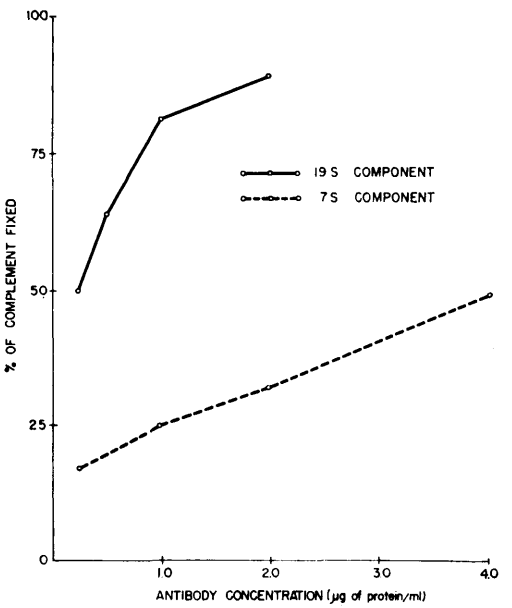

FIG. 3. Ability of antibodies anti-RKC homogenate present in 19*S* and 7*S* components to fix complement. The complexes between the antibodies and the antigen (RL homogenate at 0.55 μg/ml of the mixture) were allowed to form in the presence of complement. The percentage of complement fixed is plotted as a function of antibody concentration.

TABLE I. Properties of the 19S and 7S Components Obtained from Rabbits Anti-RKC γ-Globulins on Sephadex G-200 Gel Filtration.

Preparation injected[a]	Symptoms of APE (no. of animals with symptoms/no. of animals in group)					Presence of precipitin lines (double gel diffusion analysis)	Complement fixation
	(—)	(+)	(++)	(+++)	(++++)		
Normal rabbit serum	4/4	0/4	0/4	0/4	0/4	—	—
Rabbit anti-RKC serum	0/4	4/4	4/4	4/4	4/4	+	+
γ-Globulins anti-RKC (27.9 mg/ml)	0/4	4/4	4/4	4/4	4/4	+	Not tested
19*S* component (2.44 mg/ml)	0/4	4/4	4/4	4/4	3/4	—	+ (0.5 μg)[b]
7*S* component (7.3 mg/ml)	4/4	0/4	0/4	0/4	0/4	+	+ (4.0 μg)

[a] Amounts injected in each animal: normal rabbit serum (1.0 ml); rabbit anti-RKC serum (0.5 ml); γ-globulins anti-RKC (0.4 ml); 19*S* component (0.55 ml) and 7*S* component (0.5 ml).

[b] The values in parentheses indicate the amounts of protein to fix 50% of complement.

the antigen (control sample) and at 3, 8, and 22 days later. The control and the 3-day samples were unable to produce APE or complement fixation, whereas the 8- and 22-day samples presented both these properties. On the other hand, antibodies detectable by double-diffusion gel precipitation were only found in serum samples obtained 6–8 days after a booster with the same amount of antigen.

Discussion. In the present study, rabbit γ-globulin preparations containing antibodies to RKC or RL homogenates were separated by Sephadex G-200 gel filtration. Antibodies involved in the production of the pulmonary edema in rats were consistently found in the first peak of protein of the chromatogram (19*S* component). Associated also with this peak, there were antibodies with striking ability to fix complement when RL homogenate was used as antigen. Both these biologic properties associated with the 19*S* component disappear after reduction with 2-mercaptoethanol and alkylation with iodoacetamide.

The proteins present in the intermediate zone of the chromatogram (7*S* component), only contained antibodies against both RKC or RL homogenates when obtained from sera collected 1 week after the second injection of antigen in the course of the immunization process. These antibodies were revealed by double-diffusion gel precipitation against RKC or RL homogenates, were characterized by low ability to fix complement as compared with the 19*S* antibodies, and scarcely were able to produce symptoms of pulmonary edema.

The present investigation also defined the time course of appearance of anti-RKC antibodies involved in the production of the APE, following a single injection of RKC homogenate emulsified in complete Freund adjuvant. The responsible antibodies were present 8 days after the injection of the antigen (RKC homogenate) and reached peak titers by days 15–18. The peak titers, were again associated with the 19*S* antibodies and with the ability of these antibodies to fix complement. At this point of the immunization process antibodies against RKC or RL homogenate associated with the 7*S* components were not found.

The characteristics of this 19*S* type of antibody, are closely related to those described for the heavy antibodies raised in rabbits by intradermal inoculation with human serum albumin. Such antibodies also appear early during the immunization course, have high hemagglutinating titers and fail to precipitate

with antigen (7).

From this study, it is suggested that the ability of antibodies present in nephrotoxic sera to produce lung lesions, is associated with its complement-fixing properties. Such possibility agrees well with the previous observations that the hemolytic activity of the IgM antibodies is several times higher than that of the IgG antibodies (8).

Summary. Nephrotoxic sera were prepared in rabbits with rat kidney cortex homogenate in complete Freund adjuvant. The γ-globulins were precipitated with $(NH_4)_2SO_4$ and fractionated through gel filtration in Sephadex G-200. Antibodies responsible for the production of pulmonary edema in rat and capable of fixing complement were consistently associated with the 19*S* form of immunoglobulins; both these activities were destroyed by reduction with 2-mercaptoethanol followed by alkylation with iodoacetamide. The antibodies associated with the 7*S* immunoglobulins were revealed by gel double-diffusion analysis, possessed low capacity to fix complement, and only occasionally produced slight symptoms of pulmonary edema. The 19*S* antibodies were present in rabbit serum 8 days after the immunization and reached peak titers by days 15–18. The 7*S* antibodies, on the other hand, only were present 1 week after the booster.

1. Dias da Silva, W., Vieira, M. N. R., and Diniz, C. R. Presented: Reunião Anual da Sociedade Brasileira para o Progresso da Ciência, 20th, Sao Paulo, Brazil, 1968.

2. Wasserman, E. and Levine, L., J. Immunol. **87**, 290 (1961).

3. Ouchterlony, O., Progr. Allergy **5**, 1 (1958).

4. Lowry, O. H., Rosebrough, N. Y., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

5. Figueiredo, E. A., Gomez, M. V., Heneine, I. F., Hargreaves, F. B., and Santos, I. O., *in* "II Simpósio de Bioquímica de Planorbídeos," pp. 49-54. Curitiba Pr., Brazil (1966).

6. Reed, L. J. and Muench, H., Am. J. Hyg. **27**, 493 (1938).

7. Shulman, S., Hubler, L., and Witebsky, E., Science **145**, 815 (1964).

8. Onoue, K., Tanigaki, N., Yagi, Y., and Pressman, D., Proc. Soc. Exptl. Biol. Med. **120**, 340 (1965).

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