Immunetolerance to Experimental Autoimmune Nephrosis in Rats[1] (35015)

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In 1959 Heymann *et al.* (1) reported the experimental production of a nephrotic renal disease in rats that was believed to involve an autoimmune mechanism. Subsequently this model was identified as a membranous glomerulonephropathy (2) with a nephrotic syndrome resulting from the glomerular deposition of autologous immunecomplexes (3). The antigen has been identified as a protein, located close to the brush border of proximal convoluted tubules (4, 5) and good evidence has been obtained to support the view that an antibody to this periluminal antigen (5, 6) is the disease producing antibody. This communication describes the experimental induction of immunetolerance to this antigen.

Methods and Procedures. Random bred Sprague-Dawley male rats were used throughout. Forty-one were injected intraperitoneally (ip) with 0.05 ml of a 1:1 kidney suspension in saline starting 1 day after birth and continuing for 2 weeks, increasing the amounts slowly as tolerated to 0.6–0.8 ml by the end of the second week. From then on kidney in saline was injected once or twice weekly up to a maximum of 1 ml/injection. Injections were discontinued when the animals were 5–7 weeks of age and weighed 140–160 g. At that time, attempts were made to immunize the animals with ip injections of kidney in complete Freund's adjuvant as previously described (1). Immunization was continued until proteinuria in excess of 0.288 g/100 ml or 50 mg/24 hr appeared, at which time injections were discontinued. A maximum of 10 injections were given to rats who failed to develop proteinuria. If proteinuria failed to develop within 2 months following the tenth injection, it was assumed that no renal disease had been produced and the rats were sacrificed. Urea, creatinine, total protein and albumin, cholesterol, and total lipid concentrations were obtained from serum in all animals.

An additional 14 newborn animals received 0.05 ml of kidney–saline suspension on the first 3 days of life only, with no injection given subsequently until immunization was attempted at age 6 weeks. Other animals received the full 6 weeks neonatal pretreatment course substituting 1:1 saline suspensions of rat muscle in 24, and rat intestine in 22 prior to immunizing with rat kidney in adjuvant. Since *Mycobacterium tuberculosum* are such an important component of the Freund's adjuvant in this disease model (11), 51 newborn rats received the same neonatal pretreatment utilizing saline suspensions of either 50 or 100 mg/100 ml tubercle bacilli obtained from the same batch as those used in the subsequent sensitizing kidney in adjuvant injections of all immunized animals (3). Finally, 112 control animals received immunization with kidney in adjuvant as previously described (1) without any neonatal pretreatment.

Rat kidney, muscle, and intestine for use as antigens were obtained from saline perfused Sprague-Dawley animals and were used *in toto*; the saline suspensions of these antigens contained 51.3, 43.4, and 24.2 mg of protein/ml, respectively. All light microscopy was carried out on 3-μ sections of formalin fixed rat kidney using hematoxylin and eosin or periodic acid Schiff staining.

The globulin fractions of rabbit antirat gamma globulin and rabbit antirat beta-1-C globulin were conjugated with fluorescein

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TABLE I. Renal Disease Incidence in Sprague-Dawley Rats with and Without Neonatal Treatment with Various Tissue Suspensions.

No. of rats	Neonatal R_x		Kidney in adjuvant sensitizations no. of injections	Disease incidence (%)
	Tissues	No. of injections		
112	0	0	6.7	89.3
41	Kidney	23–27	7.4	29.2
14	Kidney	3	9.3	57.2
24	Muscle	23–27	7.7	100.0
22	Intestines	23–27	8.0	95.6
51	Tbc	23–27	8.1	76.5

isothiocyanate by methods previously described (7). All antisera, prior to conjugation, gave single lines of precipitation against normal rat serum by immunoelectrophoresis and double diffusion in agar. Serum from 48 rats was obtained prior to immunization with homologous kidneys, then sequentially after the third injection. These sera were reacted, by the indirect technique, with 2–4-μ sections of frozen unfixed normal rat kidneys and the fluorescence developed with labeled antirat gamma globulin conjugate (6). Frozen, unfixed sections of kidneys obtained from immunized animals were stained by the direct technique, using the labeled antirat gamma globulin and the antirat beta-1-C globulin conjugates (6). The specificity of the fluorescent reactions was determined by the inhibition of staining by prior exposure of the section with unconjugated serum and the absence of staining of normal tissues. Further specificity of the rabbit antirat gamma globulin was determined by the absence of staining after absorption with rat gamma globulin obtained by column chromatography.

Results. Table I shows that the incidence of renal disease produced in 112 unprepared Sprague-Dawley rats was 89.3%. The disease incidence in 41 rats that received the full neonatal treatment with rat kidney in saline was significantly reduced ($p = <0.01$) to 29.2%. Even when kidney in saline was given only on the first 3 days, the incidence was reduced to 57.2% and the number of kidney in adjuvant injections needed to produce proteinuria increased from 6.7 to 9.3. Disease incidence was not reduced when neonatal treatment was with suspensions of muscle, intestines, or tubercle bacilli. The appearance of antibody to the periluminal antigen in the sera of experimental animals is shown in Fig. 1. In 20 Sprague-Dawley rats not treated

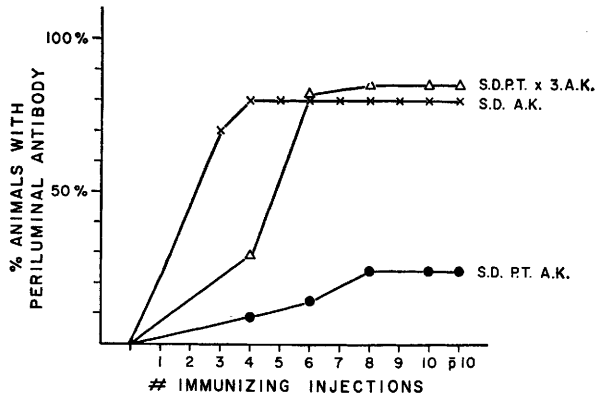

FIG. 1. Incidence with which antibody to periluminal antigen was noted in sera of serially bled Sprague-Dawley rats: (×), 20 rats without neonatal treatment with kidney; (Δ), 7 rats treated only 3 successive days after birth with kidney in saline; (●), 21 rats treated until 6 weeks of age (as described) with kidney in saline prior to sensitization with kidney in adjuvant.

neonatally with rat kidneys, circulating periluminal antibodies appeared in 70% of the sera by the third sensitizing injection and in 80% of sera by the fourth. Only 30% of the animals injected three times neonatally had circulating periluminal antibodies after the fourth injection, although this did increase to 85% by the eighth injection. Only 9% of rats receiving the full neonatal pretreatment, had this circulating antibody after the fourth injection, increasing to only 24% by the eighth injection.

Further evaluation of the sera from the 20 rats not receiving neonatal treatment demonstrated the four distinct patterns of staining previously reported (6) when reacted with normal rat kidney. In addition to the staining of the periluminal portion of the proximal tubular cells in 16 (80%), 15 (75%) demonstrated antibody to the cytoplasm of both proximal and distal tubular cells, 3 (15%) had antibody to the basal portion of proximal tubular cells, and 2 (10%) produced a sharply granular stain of the entire tubular cytoplasm. The nature and specificity of these antibodies, as well as their relationship to the disease, has been detailed previously (6). None of the sera from animals receiving neonatal treatment with saline suspensions of rat kidney demonstrated the basilar or the granular cytoplasmic staining. Only 14% of the rats receiving the full course of neonatal treatment and only 28% of rats receiving the 3 neonatal injections demonstrated diffuse cytoplasmic staining.

At the time of sacrifice the kidneys of 6 of the pretreated group of rats were studied for the presence of glomerular deposition of gamma- and beta-globulins. Two rats in which no renal disease had been produced had no demonstrable periluminal antibody in their sera, and no immune deposits were observed in their kidneys. Another 2 rats without demonstrable proteinuria demonstrated weaker, but definite, staining for periluminal antibody in their sera and had deposition of immunoglobulins in a granular pattern in the glomeruli, though not as brightly as usually seen. The sera of the 2 remaining rats with proteinuria, reacted brightly with the periluminal antigen and the kidneys contained the bright granular deposits of gamma globulin and beta-1-C in glomerular capillary loops that is characteristic of this disease model (6, 8). In the unprepared control animals, 14/20 developed proteinuria, periluminal antibody, and glomerular deposition of immunoglobulins; and 4/20 failed to develop antibody, glomerular deposits, or disease. However, 2 developed antibody in association with glomerular deposits without significant proteinuria.

Discussion. In our laboratory unprepared, random bred Sprague-Dawley rats subjected to ip injections every 2 weeks of Sprague-Dawley kidneys in Freund's complete adjuvant yielded a disease incidence of 89.3%. When treated neonatally with Sprague-Dawley kidneys in saline during the first 6 weeks prior to sensitization with kidney in adjuvants, the incidence decreased significantly to 29.2%. When injections were given after birth only on days, 1, 2, and 3, the disease incidence was 57.2%. It would appear that immunetolerance has indeed been induced in these animals, by their neonatal treatment with kidney homogenate. This is supported by the following: (i) a decreased incidence of disease that was not evident in the control groups; (ii) a delay in the onset of proteinuria in those animals developing disease; (iii) a decreased development of the disease producing periluminal antibody; (iv) delay in the appearance of this antibody in those animals that developed it. Adding further support is the decreased incidence in the expected antibodies to other renal antigens (6).

Those pretreated animals with proteinuria have developed a disease that appears immunopathogenically identical to the experimental autoimmune nephrosis in the unprepared rats, showing the same morphologic lesion by light microscopy, the same clinical syndrome, the same antibody to periluminal antigen (6) and the same glomerular deposits of gamma globulin and complement (6, 8). There also appears to be a good correlation between the appearance of periluminal antibody and the presence of the glomerular de-

positions of immunoglobulins. As has been shown earlier (6), no animal in this series developed disease in the absence of a perilumimal antibody response.

It is of interest that neonatal treatment with tubercle bacilli did not effect disease incidence. Multiple studies (10) in progress have failed to demonstrate that tubercle bacilli behave as an immunogen in the pathogenesis of this model. Their importance in adjuvant remains unquestioned, though the mode of action remains unclear.

It might be postulated that the large amount of antigen infused into these animals during the 6 weeks neonatal period persisted in sufficient quantity to prevent the appearance of disease. This could be suggested from the work of Senterfit and Germuth (9) who altered the glomerular deposition of complexes in BSA. Attempts to alter the course of autoimmune nephrosis by large daily injections of kidney antigen, after full disease had developed following immunization with kidney adjuvant, has been unsuccessful so far (12). The good correlation between circulating antibody and glomerular deposition of immunoglobulin would also indicate complexes being formed and deposited only in the presence of an antibody response. Giving only 3 newborn injections was also effective in decreasing the antibody response and the appearance of disease. Thus it would appear that immunetolerance is the underlying mechanism explaining the decreased disease incidence in neonatally treated animals.

Summary. In 112 unprepared, random bred Sprague-Dawley rats the disease incidence of experimental autoimmunenephrosis was 89.3%.

After treatment of rats in the neonatal period and up to 6 weeks of age with 1:1 rat kidney suspension in saline, the disease incidence decreased to 29.2%.

It was shown that the production of the antibody to periluminal antigen was delayed and inhibited in these animals.

It was concluded that immunetolerance had been induced in them.

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