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ANTIBODY SYNTHESIS IN KIDNEY, SPLEEN AND LYMPH NODES IN ACUTE AND HEALED FOCAL PYELONEPHRITIS

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SUMMARY.—Segmental circumscribed pyelonephritis in rabbits was associated with a significant increase in the incorporation of ^{14}C amino acids into IgG by the infected kidneys and spleen. Specific anti-*Esch. coli* antibody in the IgG class was synthesized by 8 of 10 kidneys, 5 of 10 spleens, 1 of 6 proximal lymph nodes, 1 of 3 popliteal lymph nodes and by none of the " buffy coat " cells (circulating lymphocytes). Although viable organisms were not present, non-viable antigen persisted for up to 6 months after infection. Persistent circulating antibody may arise from local antibody which is stimulated by the persistence of non-viable antigen at site of infection.

IN patients with pyelonephritis the persistence of circulating antibody to the causative organisms correlated with continued active infection within the kidney (Winberg, Anderson, Hanson and Lincoln, 1963; Vosti, Monto, and Rantz, 1965). Previous studies have demonstrated that non-viable bacterial antigen may persist within the kidney for long periods after active infection subsides (Sanford, Hunter and Donaldson, 1962; Cotran, 1963). Previously we have shown local immunoglobulin and antibody formation within the kidney during the course of experimental pyelonephritis (Lehmann, Smith, Miller, Barnett and Sanford, 1968). However, it has not been established whether circulating antibody and antibody synthesis can persist for long periods following self-limited pyelonephritis.

An evaluation of antibody synthesis in pyelonephritis was undertaken using a model in which an acute transient infection of the kidney was produced. Animals were studied at varying intervals after the active infection had subsided. This study also was designed to compare antibody synthesis in the kidney, spleen, lymph nodes and circulating lymphocytes.

MATERIALS AND METHODS

Pyelonephritis

Unilateral pyelonephritis was produced in 2–3 kg. white male New Zealand rabbits by the direct inoculation into the kidney of $1–4 \times 10^8$ *Escherichia coli* 075 (EC 075). The organisms were grown in trypticase soy broth (Baltimore Biological Laboratories, Baltimore, Maryland) for 18 hr at 37° and washed twice with sterile 0·9 per cent saline solution. After 20 mg./kg. thiamylal sodium (Surital, Parke, Davis and Company, Detroit, Michigan) and

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ether anaesthesia, under sterile technique the right kidney was exposed through a flank incision. The kidney was pierced and seared in 4 separate areas to a depth of 1 cm. with a heated 26 gauge needle and 0·1 ml. of a culture containing 10^9 organisms per ml. was injected in to each site.

Voided urine was obtained during the course of the infection and bladder urine was aspirated at death for culture. Quantitative bacterial counts were performed on tissue homogenates at death. All *Esch. coli* isolated were identified as EC 075 by the use of specific antisera (Biological Reagents Section, NCDC, Atlanta, Georgia). Then tissue was (1) placed in 10 per cent formalin for histological examination, (2) quick frozen in acetone and dry ice and stored at $-70°$ and (3) incubated to determine immunoglobulin and antibody synthesis for fluorescent antibody studies. Serum antibody levels were determined using a microtitre agglutination technique with somatic antigen as previously described (Lehmann *et al.*, 1968).

Histological Studies

Tissues fixed in 10 per cent formalin were stained with haematoxylin and eosin and examined by light microscopy.

For fluorescent antibody studies, the specimens held at $-70°$ were sectioned in a refrigerated cryostatic microtome to a thickness of approximately 4 μm. Sections were air dried, fixed in 95 per cent ethyl alcohol and washed twice with phosphate buffered saline solution (PBS) pH 7·2.

Goat anti-rabbit IgG, anti-rabbit colostral IgA and anti-rabbit IgM were prepared as described previously (Lehmann, *et al.*, 1968; and Lehmann, Smith, Miller, Barnett and Sanford, 1969). These antisera were conjugated with fluorescein isothiocyanate (FITC) (Sigma Chemical Company, St. Louis, Missouri) to prepare specific conjugates (Reisberg, Rossen and Butler, 1970). The FITC conjugates were adsorbed with a carbodiimide (Ott Chemical Company, Muskegon, Michigan) induced bentonite antigen complex containing antigen to which the antisera were prepared (*e.g.* goat anti-rabbit IgG was first adsorbed with a bentonite rabbit IgG complex). After this specific adsorption, the anti-rabbit immunoglobulin was eluted with 0·1M phthlate-HCl, pH 2·4, and dialysed against PBS. Subsequently the anti-IgG was cross-adsorbed with a bentonite rabbit colostral IgA complex and bentonite rabbit IgM complex. The anti-IgA FITC conjugate was cross adsorbed with a bentonite rabbit IgG complex and a bentonite rabbit IgM complex. The anti-IgM FITC conjugate was cross-adsorbed with a bentonite rabbit IgG and a bentonite rabbit colostral IgA complex.

Antisera to the infecting organism *Esch. coli* 075, *Esch. coli* 06, and *Esch. coli* 014 were produced by injecting rabbits every 5 days on 5 occasions with 2×10^9 boiled organisms. Antisera which contained high titre 2-mercaptoethanol resistant antibody was fluoresceinated as described above. The *Esch. coli* FITC conjugates were than specifically adsorbed with a lipopolysaccharide bentonite complex. The lipopolysaccharide was prepared by ether extraction and ethanol precipitation of cultures of the *Esch. coli* strain (Ribi, Haskins, Landy, and Milner, 1961).

The sections were stained with the specific FITC conjugates for 30 min. at room temperature in a moist atmosphere. The sections were rinsed with PBS and washed twice for 5 min. in PBS. After excess PBS was removed and the slides were blotted dry, buffered 1 per cent glycerine was dropped on to the sections and a glass cover slip applied. The slides were examined within 2 hr under a Leitz Ortholux microscope equipped with an Osram HBO200 watt high pressure mercury vapour lamp, U^{G}-1 exciter filter and a K 460 barrier filter. Controls in each experiment included the incubation of sections with fluoresceinated normal rabbit globulin and blocking of the binding of FITC conjugates of IgG, IgA and IgM by prior incubation of the tissue with the non-conjugated specific antisera.

Protein synthesis

The *in vitro* synthesis of protein and immunoglobulin was determined by incubating minced tissue in the presence of ^{14}C amino acids as described previously (Lehmann *et al.*, 1968; and Hand, Smith, Miller, Barnett and Sanford, 1970). The incubated tissue was ultracentrifuged and the soluble protein was dialysed and fractionated on DEAE cellulose. The protein fractions were dialysed against phosphate buffer, concentrated (by lyophilization) and then co-precipitated with an ovalbumin anti-ovalbumin complex to remove non-specific

radioactivity. Soluble protein was estimated by precipitation with 10 per cent trichloroacetic acid (TCA). Synthesized immunoglobulin was quantitated by immune precipitation in antibody excess using goat anti-rabbit IgG, goat anti-rabbit colostral IgA and goat anti-rabbit IgM. The precipitates were washed and dissolved in 2M NaOH (0·5 ml.) and solubilized in 1·5 ml. of BBS-2. A toulene-2,5 diphenyloxazole (PPO) scintillation cocktail (PPO 7 g. per l.) was added. Liquid scintillation counting was performed in a Beckman LS-250 spectrometer (Beckman Instruments, Fullerton, California). TCA precipitable protein and immunoglobulins were expressed as counts per min. per g. of wet weight tissue (CPM/g.). Synthesis of specific antibody was quantitated by adding 10^9 heated killed (boiled) *Esch. coli* 075 to samples of the protein fractions containing IgG. The antigen of a pseudomonas strain was used as a control.

"Buffy coat" cells were obtained by intracardiac puncture using 50 ml. syringes containing 100 units of sodium heparin and 10 ml. of a sterile 6 per cent solution of dextran in normal saline. The blood was allowed to sediment at 37° for 1 hr and the plasma containing leucocytes was collected into conical tubes. The leucocytes were washed twice with Eagle's minimal essential medium and adjusted to a concentration of 25×10^6 nucleated cells/ml. Cell suspension (1–2 ml.) were placed into disposable 25 ml. plastic tissue culture bottles (Falcon Plastics, Los Angeles, California) and 5 microcuries of ^{14}C amino acids (Schwartz BioResearch, Orangeburg, New York) were added per ml. of cell suspension. The bottles were placed horizontally in a 95 per cent O_2, 5 per cent CO_2 atmosphere and incubated at 37° for 21 hr. The incubation mixture was washed from the bottle, with normal saline and centrifuged at $12{,}000 \times g$ for 30 min. Casein hydrolysate amino acid mixture (Difco Laboratories, Detroit, Michigan) was added to the supernatant to a final concentration of 1 per cent. The final mixture was then dialysed against PBS pH 7·2. The soluble protein was co-precipitated with an ovalbumin-antiovalbumin complex and protein and immunoglobulin synthesis was determined as described above.

RESULTS

Pyelonephritis

Bacteriuria initially was present in all animals, but it cleared after 14 days. Cortical abscesses were present in the 2 animals studied at 6 and 14 days. In animals studied at later times (21–179 days) small wedge-shaped scars extending from the cortex to the medulla were present. Infiltrates consisting of polymorphonuclear leucocytes and lymphocytes were present in the animals studied at 6 and 14 days. Later, the infiltrates consisted predominantly of lymphocytes which were confined to the small scars.

Serum antibody was present by the 6th day in all animals. Two-mercaptoethanol resistant antibody to EC 075 was demonstrated in all animals by the 7th day and this persisted until the time of death.

Immunofluorescent studies

Tissue from 2 animals studied at 165 and 179 days were examined by immunofluorescence for presence of immunoglobulin and *Esch. coli* 075 antigen. Both tissues had IgG containing cells in moderate numbers. Only a few IgA containing cells were visible and no IgM cells were detected. Specific fluorescence for EC 075 was present in large clumps in both tissues throughout the scarred area. Similar fluorescence was seen with the EC 014 FITC but none was present with EC 06.

Immunoglobulin and specific antibody synthesis

IgG synthesis was significantly increased over normal in all ten kidneys studied (Table I). IgG accounted for a mean 41 per cent of the total protein synthesized by the infected kidney whereas less than 5 per cent of total protein synthesized by

the normal kidney was immunoglobulin (Table I). A significant increase in IgA synthesis was present in only 2 animals and in IgM synthesis in 3 animals.

A significant increase in total protein and IgG synthesis occurred in " pyelonephritic " spleens. Total protein and IgG synthesis in lymph nodes proximal to the kidney was comparable to that synthesized by popliteal and normal lymph nodes. The synthesis of immunoglobulins IgG, IgA and IgM expressed as a percentage of total protein synthesized was increased in the " buffy coat " cells obtained from animals with pyelonephritis.

Specific anti-*Esch. coli* antibody of IgG class was synthesized by 8 of 10 kidneys, 5 of 10 spleens, 1 of 6 proximal lymph nodes, 1 of 3 popliteal lymph nodes and by none of the " buffy coat " cells (circulating lymphocytes) studied (Table II). Specific antibody which bound to EC 014 was not present in fractions containing IgG.

TABLE I.—*Protein and Immunoglobulin Synthesis in Pyelonephritic Kidney, Spleen, Lymph Nodes and Buffy Coat Cells*

Tissue	Total protein TCA Ppt	IgG	IgA cpm/g. (Mean ± S.E.M.)	IgM	IgG	IgA	IgM
Normal							
Kidney (3)	2280 ±240	60 ±30	50 ±30	20 ±20	3	2	1
Spleen (6)	10920 ±2080	2850 ±460	290 ±110	220 ±100	26	3	2
Popliteal lymph node (3)	57290 ±27260	9460 ±2380	0 —	340 ±340	17	0	1
Buffy coat (6)	24840 ±5620	2340 ±520	930 ±110	280 ±50	9·5	3·7	1·1
Pyelonephritic							
Kidney (10)	3470 ±880	1420 ±400	120 ±70	70 ±40	41	3	2
Spleen	31470 ±6540	19520 ±3060	460 ±120	310 ±85	62	1·5	1
Proximal lymph node (6)	41200 ±3270	11640 ±3850	690 ±260	1990 ±610	28	2	5
Popliteal lymph node (3)	84920 ±7170	16870 ±9680	920 ±380	1560 ±1010	20	1	2
Buffy coat (4)	40700 ±3330	8900 ±3020	3750 ±1250	1020 ±270	22	9	3

DISCUSSION

Previous studies demonstrating local antibody synthesis within the kidney were carried out in animals challenged i.v. with organisms following transient obstruction of the ureter (Lehmann *et al.*, 1968). This method of inducing pyelonephritis resulted both in diffuse involvement of the kidney and in the exposure of extrarenal tissue to antigenic stimulation. In the current studies segmental circumscribed disease was produced in the kidney and the exposure of extra renal tissue to direct antigenic stimulation was minimal. The present studies demonstrated a significant increase in IgG synthesis within the localized area of disease whereas a significant increase in IgM and IgA synthesis was not demonstrated. The relative paucity of IgA synthesis by the localized pyelonephritic tissue is similar to the low quantities synthesized by pyelonephritic kidneys of animals with ascending infection (Hand *et al.*, 1970) and contrasts with significant synthesis of IgA by pyelonephritic kidneys following the i.v. injection of organisms (Lehman *et al.*, 1968). These data suggest that significant IgA synthesis by pyelonephritic kidneys is either

TABLE II.—*Specific IgG Antibody to* Esch. coli 075

	Right kidney			Spleen			Proximal lymph nodes			Distal lymph nodes (popliteal)			Circulating lymphocytes (buffy coat)		
Day	Specific *Esch. coli* antibody	IgG	Per cent	Specific *Esch. coli* antibody	IgG	Per cent	Specific *Esch. coli* antibody	IgG	Per cent	Specific *Esch. coli* antibody	IgG	Per cent	Specific *Esch. coli* antibody	IgG	Per cent
6	66	1540	4·3	230	3395	6·8	0	6560	0	—	—	—	0	18180	0
14	17	414	4·1	0	2280	0	152	1900	8·0	0	12920	0	—	—	—
21	0	312	0	420	28920	1·5	0	8600	0	—	—	—	—	—	—
30	15	607	2·5	0	12310	0	0	31650	0	1600	26400	6·0	—	—	—
38	340	2010	16·9	2120	18480	11·5	0	3200	0	—	—	—	—	—	—
45	137	707	19·4	59	595	10·0	—	—	—	0	310	0	0	10250	0
60	37	230	16·1	168	7790	2·2	0	7130	0	—	—	—	0	2880	0
114	0	550	0	0	820	0	—	—	—	—	—	—	0	4280	0
165	78	1110	7	0	940	0	—	—	—	—	—	—	—	—	—
179	380	3270	11·5	0	96440	0	—	—	—	—	—	—	—	—	—

related to the i.v. route of injection or to the intensity or distribution of disease.

The infected kidney and the spleen synthesized specific antibody whereas lymph nodes and circulating lymphocytes did not. Thus, lymph nodes draining a site of injection of viable organisms differ from lymph nodes draining the site of injection of protein antigens in that the latter have been demonstrated to produce specific antibody (Helmreich, Kern, and Eisen, 1962).

Although the active infections subsided spontaneously in the current experiments and viable organisms were not present, the immunofluorescent studies demonstrated the persistence of *Esch. coli* antigen within the involved areas of the kidney. These observations confirm previous studies which demonstrated that specific bacterial antigen and common antigen may persist within the kidney after the disappearance of viable organisms (Sanford *et al.*, 1962; Cotran, 1963; and Aoki, Merkel, Aoki and McCabe, 1967).

These studies also confirm the previous observations that an antiserum to common antigen is as useful in locating somatic antigen as is the antisera specific for the somatic antigen (Aoki *et al.*, 1967). The presence of this common antigen did not however stimulate the production of IgG antibody as occurred in response to the somatic antigen of the infecting organism.

The demonstration of 2-ME resistant antibody in the serum of rabbits many weeks after the initial acute infection subsides raises a question concerning previous interpretations of persistent antibody titres in man. Percival, Brumfitt and DeLouvois (1964) demonstrated that the serum antibody titres in patients with urinary tract infections fell to normal after the eradication of the infection. They postulated that persistence of the circulating antibody was associated with continued active infection. Vosti and Remington (1968) observed the persistence of 7 S haemaglutinins for at least 4 yr in the sera of patients with acute pyelonephritis although clinical relapses may have occurred in some of these patients.

The present studies favour the concept that persistent circulating antibody may reflect not only persistent active infection of the kidney but that the persistence of circulating antibody may be the consequence of the persistence of non-viable antigens at the site of previous infection. Furthermore, these data suggest that antibody which is synthesized locally within the infected kidney may contribute to that demonstrable within the circulation.

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