Clin. exp. Immunol. (1983) **53**, 562–572.

Tubulointerstitial nephritis in rabbits challenged with homologous Tamm–Horsfall protein: the role of endotoxin

E. S. BERKE, A. R. MAYRER, PEGGY MINITER & V. T. ANDRIOLE *Department of Medicine, Yale University School of Medicine, New Haven, Connecticut, USA*

(Accepted for publication 28 March 1983)

SUMMARY

Tubulointerstitial nephritis developed in 25 of 34 (74%) rabbits challenged intravenously for 2–20 weeks with adjuvant and endotoxin free homologous Tamm–Horsfall protein (THP). Lesions were characterized by focal mononuclear cellular infiltrates and microscopic scarring localized to distal nephron segments identified as thick ascending limb of the loop of Henle. Interstitial deposits of THP were found in the kidneys of more severely affected rabbits and metabolic studies demonstrated transient polyuria and tubular dysfunction. Elevations in serum IgG antibody against THP were detected in seven of 34 challenged rabbits. Tubulointerstitial nephritis was found in six of the seven rabbits with elevated antibody as well as in 19 of 27 rabbits without elevated antibody. By contrast, peripheral lymphocytes from eight of 13 rabbits with tubulointerstitial nephritis were cytotoxic against target fibroblasts in the presence of THP as compared to none of eight age matched challenged or unchallenged rabbits with normal kidneys. The presence or absence of endotoxin *in vitro* did not influence determinations of antibody- or lymphocyte-mediated cytotoxicity. These observations suggest that the tubulointerstitial nephritis which develops in rabbits challenged with THP is primarily the result of cell-mediated immune responses directed against THP, and does not require the presence of endotoxin in the challenge solution, or serum IgG antibodies directed against THP.

Keywords tubulointerstitial nephritis Tamm–Horsfall protein endotoxin cellular immunity

INTRODUCTION

Chronic tubulointerstitial nephritis (TIN) is not necessarily associated with renal infection, nor have prospective clinical studies demonstrated progressive renal damage due to bacteriuria alone (Freedman & Andriole, 1974; Sanford, 1975). Clinical and experimental evidence has indicated that other factors such as vesicoureteral reflux (VUR) or obstruction may cause chronic TIN even in the absence of infection (Hutch & Smith, 1969; Bailey, 1973; Salvatierra, Kountz & Bellzer, 1973; Hodson *et al.*, 1975). Therefore, recent studies have examined the role of normal urinary constituents, such as Tamm–Horsfall protein (THP), in the pathogenesis of reflux associated TIN. Both abnormal extra-tubular deposits of THP in association with TIN (Solez & Heptinstall, 1978; Zager, Cotran & Hoyer, 1978; Resnick, Sisson & Vernier, 1978; Bhagavan, Wenk & Dutta, 1979) and abnormal levels of serum antibodies directed against THP (Hanson, Fasth & Jodal, 1976; Fasth, Hanson & Asscher, 1977; Marier *et al.*, 1978; Fasth *et al.*, 1981) have been found in bacteriuric patients with reflux or obstruction. These observations have suggested that chronic TIN may be, in part, an immunological lesion triggered by THP.

Correspondence: Dr A.R. Mayrer, Department of Internal Medicine, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510, USA.

We have recently shown (Mayrer *et al.*, 1982) that rabbits injected intravenously with homologous urine or purified rabbit THP develop focal lesions of TIN localized to the thick ascending limb of the loop of Henle, a site normally rich in endogenous THP (Hoyer, Sisson & Vernier, 1979; Sikri *et al.*, 1981). We implicated an important pathogenetic role for cell-mediated immune responses against THP in this model. Although this as well as similar evidence in a rat model (Hoyer, 1980) demonstrated that TIN may involve non-bacterial antigens such as THP, the role of bacteriuria in the pathogenesis of reflux associated TIN has not been totally defined. Since bacteriuria is almost always caused by endotoxin containing micro-organisms, the present study was undertaken to evaluate the role of endotoxin in the pathogenesis of TIN in our rabbit model. Therefore, the present study describes the production of TIN in rabbits challenged with endotoxin free homologous THP, examines the associated cellular and humoral immune responses to endotoxin free THP, and compares them to the results observed with THP containing endotoxin.

MATERIALS AND METHODS

Tamm–Horsfall protein. Endotoxin free rabbit THP was prepared from sterile, pooled New Zealand white rabbit urine (Pel Freeze Biologicals, Rogers, Arkansas, USA) as previously described (Mayrer *et al.*, 1982) with the following modifications: (1) sterile technique was used throughout; (2) all solutions were pyrogen free; (3) all glassware was rendered endotoxin free by heating at 150°C for 3 h and (4) the product was frozen at −70°C in pyrogen free distilled water. Endotoxin free THP produced negative results in the limulus lysate assay (Elin *et al.*, 1975) at 1, 4 and 24 h. Rabbit THP prepared without special attention to endotoxin contamination contained 0·3% of limulus lysate positive material. Both preparations demonstrated a characteristic protein band at 105,000 daltons on polyacrylamide gel electrophoresis in the presence of SDS. Endotoxin free THP also contained several small molecular weight bands after storage at −70°C for 6 months. The two preparations bound equally well to anti-rabbit THP by solid phase radioimmunoassay.

Experimental model. Thirty-four New Zealand white rabbits were injected i.v. with endotoxin free THP (150 μg/ml) as previously described (Mayrer *et al.*, 1982). Briefly, 6·0 ml was given in divided doses over the first 3 days and subsequent boosts were given every 28 days (0·8 ml in four divided doses). No adjuvants were used. Rabbits were bled by cardiac puncture at 0, 2, 4, 8, 12, 16 and 20 weeks following initial challenge. Serum was stored at −70°C for serum anti-THP antibody determinations and heparinized blood was processed within 24 h for lymphocyte (LC) studies. Some rabbits were sacrificed at each of these intervals for renal histopathology studies. The results in these rabbits were compared with previous observations in 11 rabbits challenged with endotoxin containing THP. In addition, five age matched unchallenged rabbits served as normal controls.

Renal function studies. Renal function was evaluated sequentially (baseline, 1, 3, 6, 10, 14, 18 weeks) in challenged rabbits and in three age matched normal controls. Rabbits were placed in metabolic cages for 72 h during which three successive 24 h urines were collected. For the first 24 h, rabbits received known quantities of water and food *ad libitum*. For the second and third collections, water and food were withheld. Pitressin tannate in oil (0·1 u vasopressin, Parke-Davis, Detroit, Michigan, USA) was administered prior to the final collection. Urine osmolality was measured with a thermoelectric osmometer (Advanced Instruments, Inc., Newton, Maine, USA). Urine and serum creatinine concentrations were determined and creatinine clearances (ml/min) were calculated by the following formula:

$$\frac{\text{urine creatinine (mg\%)}}{\text{Serum creatinine (mg\%)}} \times \frac{\text{24 h urine volume (ml)}}{\text{1440 (min)}}.$$

Histology. Kidneys were removed at sacrifice and transverse sections from the mid-portion were stained with haematoxylin & eosin, periodic acid Schiff (PAS), and Masson's as previously described (Mayrer *et al.*, 1982). Coded sections were examined independently by the authors and graded on a semiquantitative basis: 0, no cellular infiltrates; 1+, definite but rare focal infiltrates (one to four per section); 2+ moderate focal infiltrates (five to 10) with scarring; and 3+, numerous (> 10) focal infiltrates with extensive microscopic scarring. Immunohistochemical staining for THP

by the peroxidase–anti-peroxidase (PAP) method to identify the thick ascending limb of the loop of Henle (Hoyer *et al.*, 1979) was kindly performed by Dr C.J. Hodson and staff.

Anti-THP antibody. Serum IgG antibody to rabbit THP was measured by a radioimmunoassay which detects binding of specific antibody to immobilized antigen (endotoxin free THP or endotoxin contaminated THP, 32 μg/ml) as traced by ^{125}I-labelled staphylococcal protein A (Marier, Jansen & Andriole, 1979). Baseline serums obtained from 34 rabbits prior to challenge and three normal, unchallenged control rabbits provided the normal antibody range ($\leq$0·4 μg/ml). Serums which bound ^{125}I-protein A to solid phase THP by more than two standard deviations (s.d.) above the mean of normals were considered positive. Simultaneous antibody determinations were repeated on 11 serums obtained 12 months previously (stored at −70°C) from rabbits challenged with endotoxin-containing THP.

Studies of cell-mediated immunity. Cell-mediated immunity was assessed by peripheral LC-induced cytotoxicity against mouse A9 fibroblast target monolayers in the presence of purified antigen as previously described (Mayrer *et al.*, 1982). All tests were performed in 0·2 ml of minimal essential medium (MEM-E) (GIBCO Laboratories, Grand Island, New York, USA) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, McLean, Virginia, USA) 2mM/ml glutamine, 50 u/ml penicillin and 50 μg/ml streptomycin (GIBCO), in flat bottom tissue culture plates. Briefly, 2×10^5 LC were added to established fibroblast monolayers and incubated at 37°C in 5% CO_2 for 72 h in the presence or absence of antigens or mitogens. Surviving target cells were enumerated, and cytotoxicity was calculated as the percentage reduction in targets incubated with LC plus antigen as compared to targets incubated with LC alone. All tests were performed in duplicate or triplicate. Target cell loss which exceeded that of control LC's by 2 s.d. was considered significantly elevated. The antigens studied were endotoxin free rabbit THP and endotoxin contaminated rabbit THP (0·3% endotoxin), which were diluted in supplemented MEM-E and added to obtain tissue culture concentrations of 10 μg/ml. Phytohaemagglutinin (PHA, Sigma Chemical Co., St Louis, Missouri, USA) was used as a positive mitogenic control (10 μg/ml).

Statistical methods. Frequency differences between groups of rabbits were compared by Chi-square analysis with Yates correction and differences between group means were analysed by Student's *t*-test.

RESULTS

Histopathology

Focal TIN was found in 25 of 34 (74%) rabbits challenged with endotoxin free rabbit THP. Lesions appeared as early as 2 weeks after initial challenge and were present in 20 of 29 (69%) studied from 2 to 12 weeks, in five of five at $\geq$ 16 weeks, but in none of three normal controls. No clear relationship was evident between duration of challenge and magnitude of TIN (Table 1). Lesions were confined to the corticomedullary junction in the kidneys of all affected rabbits. The smallest lesions consisted of focal mononuclear cellular infiltrates which encircle isolated segments of the thick ascending limb of the loop of Henle. Involved tubular cells frequently exhibited evidence of injury such as cell swelling and indistinct nucleoplasm (Fig. 1a). Fibroblast proliferation and peritubular collagen containing deposits were detected in all such foci seen in sections stained with Masson's (Fig. 1b).

In rabbits with five or more foci of TIN per section (2+, 3+) the cellular infiltrates were larger and often abutted adjacent straight segments of the proximal tubules. Coalescence of discrete foci into larger infiltrates was frequently seen, usually extending within medullary rays (Fig. 2). PAP stains in five of five rabbits studied with 2+ or 3+ TIN demonstrated discreet interstitial deposits of THP within foci of intense cellular infiltration. Furthermore, the THP deposits were found adjacent to injured or disrupted tubules. Glomeruli, arcuate veins and venous channels, arterioles and collecting ducts appeared normal in all rabbits. Polymorphonuclear leucocytes were not detected in lesions at any time.

Renal function

Serial serum and urine creatinine concentration, creatinine clearance (CrCl), basal water intake and urine output, and urinary osmolality were obtainable in 19 of 34 challenged rabbits and in three

Table 1. Tubulointerstitial nephritis in rabbits challenged with endotoxin free homologous Tamm–Horsfall protein

Challenge duration (weeks)	Frequency of TIN*	Grade of TIN†			
		O	1+	2+	3+
2	4/7	3	0	2	2
4	5/6	1	2	1	2
8	6/8	2	3	3	0
12	5/8	3	2	1	2
16	3/3	0	1	0	2
20	2/2	0	1	0	1
Total	25/34				

* Number of rabbits with tubulointerstitial nephritis (TIN) per number studied at the intervals indicated.

† 0, normal; 1+, one to four focal infiltrates per section; 2+, five to 10 infiltrates with scarring; 3+, >10 infiltrates with scarring.

unchallenged, age matched controls. The results of these studies were evaluated according to the severity of TIN: six of the 19 challenged rabbits demonstrated normal kidneys at sacrifice, five had 1+ TIN, three had 2+ TIN and five had 3+ TIN. After 1 week of challenge significant abnormalities in renal function were observed for the five rabbits with 3+ TIN when compared to the remaining 17 rabbits (14 challenged and three control) with either no or lesser degrees of TIN. Specifically, Table 2 shows that these 3+ TIN rabbits demonstrated elevated serum creatinine and urinary output as well as depressed urinary osmolality at 1 week of challenge.

A persistently elevated serum creatinine level was observed in rabbits with severe TIN (3+) at 3 weeks of challenge ($1{\cdot}36 \pm 0{\cdot}11$ mg%) as compared to 13 other challenged and unchallenged rabbits ($1{\cdot}13 \pm 0{\cdot}10$ mg%, $P < 0{\cdot}01$). Throughout the remainder of the 18 week protocol, maximum urinary concentration ability was marginally lower for 3+ TIN rabbits, but the difference was only significant at 14 weeks. Specifically, three 3+ TIN rabbits demonstrated a mean urinary osmolality of $1{,}162 \pm 273$ mOsm/l after vasopression as compared to $1{,}616 \pm 183$ mOsm/l for five remaining challenged and unchallenged rabbits ($P < 0{\cdot}05$).

Rabbit anti-THP antibody formation

Elevated serum levels of anti-THP IgG antibody were detected in seven of 34 (20·6%) rabbits challenged with endotoxin free THP (range 0·4–2·0 μg/ml) as compared to none of 34 prior to challenge and none of three age matched unchallenged controls. Antibody elevations were detected equally well with either endotoxin free or endotoxin contaminated rabbit THP as the solid phase radioimmunoassay antigen. Once elevated, serum antibodies remained abnormal at the time of sacrifice in five rabbits (three with 3+ TIN, one with 2+ TIN and one with normal kidneys) and returned to normal after a single, transient elevation in two rabbits (both with 3+ TIN) during continued challenge. TIN was found in six of these as well as in 19 of 27 rabbits without elevated antibody levels ($P > 0{\cdot}7$).

Kinetics of cell-mediated immunity to THP

Peripheral LC obtained from 16 challenged and five age matched normal control rabbits were studied for LC-mediated cytotoxicity. In the presence of endotoxin free THP, the mean cytotoxicity (% ± s.e.) for 13 challenged rabbits with TIN ($14{\cdot}8 \pm 3{\cdot}9$%) was significantly greater than that found

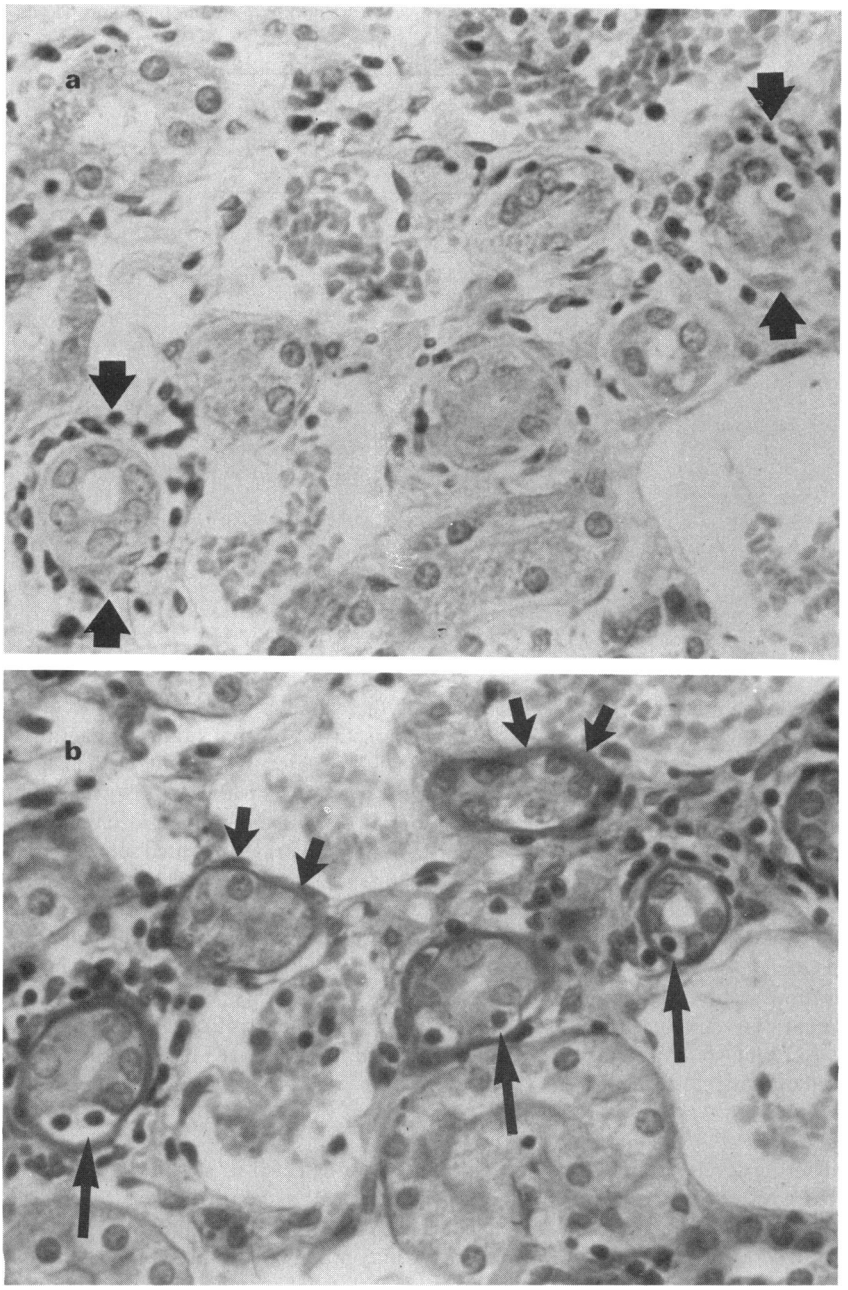

Fig. 1. (a) Appearance of focal tubulointerstitial nephritis in a rabbit challenged with endotoxin free rabbit Tamm–Horsfall protein (THP) for 12 weeks. A mononuclear cellular infiltrate is localized to several segments of the thick, ascending limb of the loop of Henle (arrows) identified by peroxidase–anti-peroxidase staining for THP (counterstained lightly with haematoxylin & eosin; magnification × 500). (b) Serial section from the kidney of rabbit described in (a) stained with periodic acid Schiff. Mononuclear cells which have infiltrated between tubular epithelial cells of involved segments are evident (large arrows). In this section the concentric thickenings (small arrows) represent basilar fibrosis as identified by Masson's staining (magnificantion × 500).

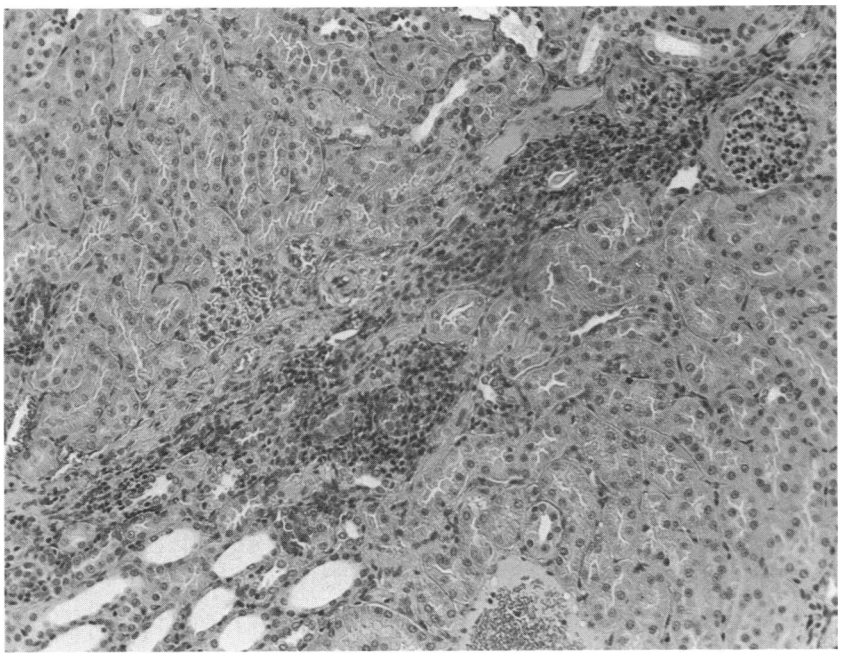

Fig. 2. Appearance of focal, tubulointerstitial nepthritis extending within a medullary ray from the corticomedullary junction (lower left) in a rabbit challenged with endotoxin free THP for 16 weeks (haematoxylin & eosin, magnification × 125).

for normal control rabbits ($1{\cdot}2 \pm 1{\cdot}2\%$, $P < 0{\cdot}05$). Three other challenged rabbits demonstrated normal kidneys but none had detectable LC-mediated cytotoxicity. When endotoxin contaminated THP was used as the *in vitro* antigen, similar LC-mediated cytotoxicity was found. The following correlation was noted for 24 paired determinations (endotoxin free *vs* endotoxin contaminated THP as *in vitro* antigen) in 13 rabbits: $y = 0{\cdot}712x + 6{\cdot}4\%$, $r = 0{\cdot}549$, $P < 0{\cdot}01$). Better than 90% agreement was found between the two THP antigens in defining LC-mediated cytotoxicity of individual rabbits as abnormal or normal.

Table 3 shows the range of endotoxin free THP cytotoxicity values for challenged rabbits in relation to duration of challenge and magnitude of TIN. Abnormal cytotoxicity ($> 6{\cdot}6\%$) was found in eight of the 13 challenged rabbits with TIN (two of five with 1+ TIN and three of four each with 2+ and 3+ TIN) as compared to none of the challenged rabbits with normal kidneys and none of the normal unchallenged controls. Three of four rabbits with 3+ TIN demonstrated elevations in serum THP antibody at the same time as abnormal LC cytotoxicity. By contrast, only one of nine rabbits with 1+ or 2+ TIN and none of the rabbits with normal kidneys (three challenged and five control) demonstrated simultaneous abnormalities in serum antibody and LC cytotoxicity. No differences in the PHA-induced cytotoxic responses between LC from challenged and unchallenged control animals were found. The mean PHA cytotoxicity for 10 determinations in five normal controls was $40{\cdot}2 \pm 7{\cdot}8\%$ as compared to $44{\cdot}3 \pm 3{\cdot}3\%$ for 26 determinations in 16 challenged rabbits. PHA cytotoxicity was not influenced by duration of challenge, magnitude of TIN, or presence of serum THP antibody.

Comparison of TIN in THP challenged rabbits with/without contaminating endotoxin

Table 4 summarizes the histological outcome and immune responses to THP in the present study as compared to our previously published series (Mayrer *et al.*, 1982). The respective frequency of TIN ($73{\cdot}5\%$ and $72{\cdot}7\%$), mean duration of challenge prior to sacrifice ($9{\cdot}0 \pm 1{\cdot}1$ and $8{\cdot}8 \pm 1{\cdot}9$ weeks), median (8 weeks) and range (2–20 and 2–16 weeks) were similar for rabbits challenged up to 20 weeks with endotoxin free or endotoxin containing THP. A highly significant difference in the

Table 2. Renal function abnormalities in rabbits challenged with endotoxin free Tamm–Horsfall protein

	1 week before challenge	1 week after challenge		P†
		Normal, * 1+, 2+ TIN	3+ TIN	
Serum creatinine (mg%)	1·18±0·20‡ $n=22$§	1·27±0·14 $n=17$	1·54±0·15 $n=5$	<0·01
Urine creatinine (mg%)	72·1±30·3 $n=20$	73·0±34·6 $n=15$	41·4±6·1 $n=5$	NS
CrCl (ml/min)	6·43±1·89 $n=20$	5·02±1·50 $n=15$	4·47±0·57 $n=5$	NS
Water intake (I) (ml/day)	283±100 $n=20$	299±110 $n=15$	407±121 $n=5$	NS
Urine output (O) (ml/day)	179±67 $n=20$	133±57 $n=15$	242±43 $n=5$	<0·01
I-O (ml/day)	98±80 $n=20$	166±71 $n=15$	165±80 $n=5$	NS
Urine osmolality (mOsm/l)				
Basal	N.T.¶	855±350 $n=17$§	522±156 $n=5$	NS
Dehydration	N.T.	1,358±262 $n=12$	886±309 $n=4$	<0·01
Dehydration plus vasopressin	N.T.	1,370±289 $m=12$	943±227 $n=4$	<0·01

* Includes three unchallenged age matched control rabbits.

† Level of significant difference between two groups 1 week after challenge as determined by Student's t-test. NS = not significant ($P>0·05$).

‡ ±1 standard deviation.

§ Number of rabbits studied; two rabbits excluded from creatinine and water balance studies, and six rabbits excluded from urinary osmolality studies because of failure to void during one or more 24 h collection periods.

¶ Not tested.

frequency of elevated serum anti-THP antibodies was observed between these two THP challenged groups with comparable TIN (six of 25 as compared to seven of eight, respectively, $P<0·01$). The latter results are from repeat determinations performed simultaneously during the present study on specimens frozen for 12 months, and were in agreement with the initial determinations.

DISCUSSION

We have found that rabbits challenged for up to 20 weeks with endotoxin free rabbit THP developed a characteristic TIN with microscopic scarring and renal tubular dysfunction. Abnormal peripheral LC-mediated cytotoxic responses in the presence of THP occurred in association with the development of these lesions. In contrast, only infrequent elevations in serum antibody directed against THP were found.

The nature of these lesions as well as their variable onset and severity were identical to those produced in rabbits injected with homologous urine or rabbit THP containing endotoxin (Mayrer *et al.*, 1982). TIN, in the present study, was also characterized by focal collections of predominately small LC localized by immunohistochemical staining to thick, ascending limbs of the loop of Henle, one of the distal tubular segments where endogenous THP is found (Hoyer *et al.*, 1979; Sikri *et al.*, 1981). Also comparable was the extension of infiltrates in a cortical direction, in association with medullary rays, in rabbits with 2+ and 3+ TIN. Fibrosis invariably accompanied both limited and

Table 3. Lymphocyte-mediated cytotoxicity in rabbits challenged with endotoxin free homologous Tamm–Horsfall protein

Protocol duration (weeks)	Unchallenged normal controls	Grade of lesion in challenged rabbits			
		0	1+	2+	3+
2	0%*	0,0	—	—	22§
4	0	0	3	11	—
8	6	—	0	27,0	—
12	0	—	44	23§	—
16	0	—	19	—	31§, 10§
20	—	—	3	—	0
	1·2±1·2†	0±0	13·8±8·2	15·2±6·1	15·8±6·8
	0/5‡	0/3	2/5	3/4	3/4

* Percentage cytotoxicity in each animal studied. Values were derived as the proportional reduction in target fibroblast survival by lymphocytes plus endotoxin free THP as compared to by lymphocytes alone. Values >6·6% exceeded the mean of normal control lymphocytes by more than 2 s.d. and were considered abnormal. (§) Rabbits (4) with elevated serum levels of anti-THP antibody.

† Mean cytotoxicities ± 1 s.e.

‡ Number abnormal (>6·6%) per number studied.

Table 4. The relationship of tubulointerstitial nephritis, serum antibody to THP, and lymphocyte-mediated cytotoxicity in rabbits challenged with endotoxin free or endotoxin containing THP for up to 20 weeks

Endotoxin free THP challenge group ($n=34$)	Elevated serum THP antibody	Cytotoxic lymphocytes	Both findings
TIN ($n=25$) (9·0±1·1)‡	6/25*	8/13	4/13†
Normal kidneys ($n=9$) (6·9±1·5)	1/9	0/3	0/3
	$P<0·01$	$P>0·10$	$P<0·05$
Endotoxin containing THP challenge group ($n=11$)§			
Tin ($n=8$) (8·8±1·9)	7/8	8/8	7/8
Normal kidneys ($n=3$) (10·0±4·2)	1/3	2/3	1/3

* Number of rabbits with histological or immunological finding per number studied in THP challenge groups indicated.

† Includes three of four rabbits with 3+ TIN and one of nine rabbits with 1+ or 2+ lesions of TIN.

‡ Mean duration (weeks ± s.e.) of challenge at the time of study.

§ Rabbits from previously published series (Mayrer *et al.*, 1982).

extended foci of lymphocytic infiltration. In the present study TIN was not observed in age matched unchallenged control rabbits. We have previously shown that TIN does not occur in other age matched unchallenged rabbits as well as in rabbits challenged with THP depleted urine (Mayrer *et al.*, 1982).

The present studies have demonstrated renal tubular dysfunction in rabbits with the severest grade of TIN (3+). Specifically, a relative polyuria was found early in association with decreased urinary concentration ability. Similar concentrating defects were also evident as late as 14 weeks. Elevations in serum creatinine (at 1 and 3 weeks) and a trend toward reduced creatinine clearances in 3+ TIN rabbits may have resulted from compensatory haemodynamic changes, since glomerular injury was not detected. Rapid compensatory changes in plasma and total blood volume, extracellular water and renal handling of water have been well described in the rabbit (Courtice & Gunton, 1949; Neuberger & Niven, 1951).

The finding of small interstitial aggregates of THP in the kidneys of rabbits with 2+ and 3+ TIN is a new and important observation in this model since similar deposits of THP have been found in 25–50% of human kidneys with chronic TIN (Zager *et al.*, 1978; Resnick *et al.*, 1978). As in the human studies, the THP deposits in our rabbits were associated with areas of mononuclear cellular infiltration as well as with areas of disrupted tubules. Since the smallest and presumably the earliest, areas of focal TIN were not associated with interstitial THP deposits, it appeared likely that the deposits were secondary to tubular disruption. Such deposits of antigenic material, nevertheless, might function to propagate an established TIN.

The humoral immune response to THP appeared blunted in the present study since only six of 25 rabbits with TIN developed elevations in anti-THP antibody as compared to seven of eight re-tested rabbits challenged with endotoxin containing THP. The two THP preparations were comparable in concentration, binding affinity to specific antibody, and electrophoretic appearance. Also, the challenge protocols were the same. This suggests that the absence of endotoxin (< 10 pg/ml) from THP used for injections accounted for the infrequent development of antibodies directed against THP. This would not be unexpected since bacterial endotoxins may have profound effects on humoral responses. Specifically, augmented antibody production has been found when endotoxins are given simultaneously with specific antigen (Chester, DeClercq & Merigan, 1971; Mond, Kim & Siskind, 1974) or when endotoxic glycolipid (lipid A) is complexed with antigen to create an adjuvant effect (Schenck *et al.*, 1969). Endotoxins may also augment antibody responses to native host proteins, e.g. anti-DNA antibodies and anti-IgG antibodies in murine models (Fournier & Miescher, 1974; Izui, Eisenberg & Dixon, 1979). Finally, studies in rabbits demonstrated that endotoxin may initially suppress humoral responses to an antigen, yet prime the animal for subsequent boosters with the same antigen (Whang & Neter, 1967).

The present observations further support the role of cell-mediated immunity to THP in the pathogenesis of THP-induced TIN in the rabbit. More than 60% of rabbits with TIN demonstrated abnormal LC-mediated cytotoxicity against target fibroblasts in the presence of endotoxin free THP. This finding was specific for rabbits with TIN, was not associated with alterations in mitogenic (PHA) responsiveness, and was not influenced by endotoxin in the *in vitro* antigen. Furthermore, the magnitude and prevalance was the same as that found for the LC of rabbits challenged with endotoxin containing THP. Therefore, it appears unlikely, from our studies in this rabbit model, that contaminating endotoxins directly modified either *in vitro* or *in vivo* manifestations of cellular immunity such as have been observed in other systems (Gery, Gershon & Waksman, 1971; Narayanan, Kloehn & Sundharadas, 1978). We have previously demonstrated that one mechanism of rabbit LC-mediated cytotoxicity against target fibroblasts involves the release of soluble factors, possibly lymphokines, by LC upon re-exposure to the sensitizing antigen (Mayrer *et al.*, 1982). A renotropic migration of such sensitized peripheral LC to the distal nephron segments rich in endogenous THP is one possible *in vivo* mechanism of focal TIN in our model. A similar mechanism has been suggested in another model by Neilson & Phillips (1979).

Serum anti-THP antibodies also did not appear to be required for the development of TIN, but were associated with abnormal cytotoxic LC in those rabbits with the most severe lesions and tubular dysfunction (3+). This supports the possibility that anti-THP antibodies, in the absence of renal immunoglobulin deposition in this model (Mayrer *et al.*, 1982), may enhance antigen

recognition by sensitized effector LC *in vivo* through an 'arming mechanism', a loss of self-tolerance, or by antibody-dependent cell-mediated cytotoxicity. Although the present study has not explored these potential mechanisms of antibody participation, Lagrange has shown that one effect of basterial endotoxin on cell-mediated immunity is indirectly mediated through endotoxin's effect on humoral immunity (Lagrange *et al.*, 1975).

Mechanistic relationships such as these may be important in experimental as well as clinical TIN where multiple, and possibly interdependent, pathogenetic factors may be present, e.g. bacteriuria, obstruction, VUR. Recent studies have suggested the existence of common antigenic determinants between non-bacterial and bacterial antigens in the pathogenesis of chronic TIN. In particular, antigenic cross-reactions have been demonstrated between human THP and soluble veronal extracts of *E. coli* and other coliforms (Fasth *et al.*, 1980; Mayrer, Miniter & Andriole, 1982). We specifically sought, but did not detect *in vitro* evidence of such cross-reactions during our studies of THP-induced rabbit TIN. However, our rabbits did not have bacteriuria, which would be a potential source of bacterial endotoxin and/or bacterial proteins in patients with TIN who have urinary tract infections and reflux or obstruction.

We have demonstrated that systemic injections with adjuvant and endotoxin free THP produced TIN in the rabbit. We suggest that the focal lesions are predominately cell-mediated and directed at tubular epithelial sites of endogenous THP or interstitial deposits of THP. Endotoxin was not required for the production of these lesions, but may enhance the elaboration of serum IgG antibodies directed against THP. Although antibodies were also not required for the initiation of TIN, it is possible that they contributed to the development of the most severe lesions. A comparable cell-mediated immune response to human THP might be immunopathogenic in chronic TIN due to urinary reflux or obstruction in which extratubular extravasation of THP occurs into the interstitium and venous and lymphatic channels. In addition, intermittent bacteriuria might provide sufficient endotoxin to enhance the host's autoimmune responses to THP. The present observations suggest that further studies of the effect of endotoxins on humoral responses to THP, as well as the relationship of these antibodies to cellular responses to THP may provide a clearer understanding of the pathogenesis, prevention, early diagnosis and treatment of chronic TIN.

The authors gratefully acknowledge the contributions of Drs F.F. Richards, C.J. Hodson, J. Ryan and H. Malech, and the expert assistance of Susan Twohill, M. Shachner, Susan Marino and Dorothy Nitshke. These studies were supported in part by NIADDKD grants Nos AM00836, AM27797, and NIH BRSG No. RR05358. Dr Mayrer is a George A. Carden Scholar of the Romill Foundation.

REFERENCES

BAILEY, R.R. (1973) The relationship of vesicoureteric reflux to urinary tract infections and chronic pyelonephritis—reflux nephropathy. *Clin. Nephrol.* **1,** 132.

BHAGAVAN, B.S., WENK, R.E. & DUTTA, D. (1979) Pathways of urinary backflow in obstructive uropathy. *Hum. Pathol.* **10,** 669.

CHESTER, T.J., DECLERCQ, E. & MERIGAN, T.C. (1971) Effect of separate and combined injections of poly rI: poly rC and endotoxin on reticuloendothelial activity, interferon, and antibody production in the mouse. *Infect. Immun.* **3,** 516.

COURTICE, F.C. & GUNTON, R.W. (1949) The determination of blood volume by the carbon monoxide and dye (T-1824) methods in rabbits. *J. Physiol.* **108,** 405.

ELIN, R.J., ROBINSON, R.A., LEVINE, A.S. & WOLFF, S.M. (1975) Lack of clinical usefulness of the limulus test in the diagnosis of endotoxemia. *N. Engl. J. Med.* **293,** 521.

FASTH, A., HANSON, L.A. & ASSCHER, A.W. (1977) Autoantibodies to Tamm–Horsfall protein in detection of vesicoureteric reflux and kidney scarring. *Arch. Dis. Child.* **52,** 560.

FASTH, A., AHLSTEDT, S., HANSON, L.A., JANN, B., JANN, K. & KAIJSER, B. (1980) Cross-reactions between the Tamm–Horsfall glycoprotein and *Escherichia coli*. *Int. Arch. Allergy Appl. Immun.* **63,** 303.

FASTH, A., BENGTSSON, U., KAIJSER, B. & WIESLANDER, J. (1981) Antibodies to Tamm–Horsfall protein associated with renal damage and urinary tract infections in adults. *Kidney Int.* **20,** 500.

FOURNIER, G.J. & MIESCHER, P.H. (1974) Release of DNA in circulating blood and induction of anti-DNA antibodies after injection of bacterial lipopolysaccharides. *J. exp. Med.* **140,** 1189.

FREEDMAN, L.R. & ANDRIOLE, V.T. (1974) A long-term study of women with urinary tract infections.

Proc. 5th Internat, Congr. Nephrol., Mexico, 1972. **3,** 230.

GERY, I., GERSHON, R.K. & WAKSMAN, B.H. (1971) Potentiation of cultured mouse thymocyte responses by factors released by peripheral lymphocytes. *J. Immunol.* **107,** 1778.

HANSON, L.A., FASTH, A. & JODAL, U. (1976) Autoantibodies to Tamm–Horsfall protein, a tool for diagnosing the level or urinary tract infection. *Lancet*, **i,** 226.

HODSON, C.J., MALING, T.M.J., MCMANAMON, P.J. & LEWIS, M.G. (1975) The pathogenesis of reflux nephropathy (chronic atrophic pyelonephritis). *Br. J. Radiol.* **(Suppl. 13)** 1.

HOYER, J.R. (1980) Tubulointerstitial immune complex nephritis in rats immunized with Tamm–Horsfall protein. *Kidney Int.* **17,** 284.

HOYER, J.R., SISSON, S.P. & VERNIER, R.L. (1979) Tamm–Horsfall glycoprotein: ultrastructural immunoperoxidase localization in rat kidney. *Lab. Invest.* **41,** 168.

HUTCH, J.A. & SMITH, D.R. (1969) Sterile reflux: report of 24 cases. *Urol. Int.* **24,** 460.

IZUI, S., EISENBERG, R.A. & DIXON, F.J. (1979) IgM rheumatoid factors in mice injected with bacterial lipopolysaccharides. *J. Immunol.* **122,** 2096.

LAGRANGE, P.H., MACKANESS, G.B., MILLER, T.E. & PARDON, P. (1975) Effects of bacterial lipopolysaccharide on the induction and expression of cell-mediated immunity. I. Depression of the efferent arc. *J. Immunol.* **114,** 442.

MARIER, R., FONG, E., JANSEN, M., HODSON, C.J., RICHARDS, F. & ANDRIOLE, V.T. (1978) Antibody to Tamm–Horsfall protein in patients with urinary tract obstruction and vesicoureteral reflux. *J. infect. Dis.* **138,** 781.

MARIER, R., JANSEN, M. & ANDRIOLE, V.T. (1979) A new method for measuring antibody using radiolabelled Protein A in a solid phase radioimmunoassay. *J. Immunol. Meth.* **28,** 41.

MAYRER, A.R., KASHGARIAN, M., RUDDLE, N.H., MARIER, R., HODSON, C.J., RICHARDS, F.F. & ANDRIOLE, V.T. (1982) Tubulointerstitial nephritis and immunologic responses to Tamm–Horsfall protein in rabbits challenged with homologous urine or Tamm–Horsfall protein. *J. Immunol.* **128,** 2634.

MAYRER, A.R., MINITER, P. & ANDRIOLE, V.T. (1982) Antigenic cross-reactions between Tamm–Horsfall protein and pathogenic coliforms. *Clin. Res.* **30,** 374A, (Abstract).

MOND, J., KIM, Y.T. & SISKIND, G.W. (1974) Studies on the control of antibody synthesis. V. Effect of non-specific modification of the magnitude of the immune response on the affinity of the antibody synthesized. *J. Immunol.* **112,** 1255.

NARAYANAN, P.R., KLOEHN, D.B. & SUNDHARADAS, G. (1978) Immune response to alloantigens *in vitro*, amplification of the development of cytotoxic T lymphocytes by lipopolysaccharide and polyadenylic: polyuridylic acid. *J. Immunol.* **121,** 2502.

NEILSON, E.G. & PHILLIPS, S.M. (1979) Cell-mediated immunity in interstitial nephritis. II. T lymphocyte effector mechanisms in nephritis guinea pigs: analysis of the renotropic migration and cytotoxic response. *J. Immunol.* **123,** 2381.

NEUBERGER, A. & NIVEN, J.S.F. (1951) Haemoglobin formation in rabbits. *J. Physiol.* **112,** 292.

RESNICK, J.S., SISSON, S. & VERNIER, R.L. (1978) Tamm–Horsfall protein: abnormal localization in renal disease. *Lab. Invest.* **38,** 550.

SALVATIERRA, O., KOUNTZ, S.L. & BELLZER, F.O. (1973) Primary vesicoureteral reflux and end-stage renal disease. *JAMA*, **226,** 1454.

SANFORD, J.P. (1975) Urinary tract symptoms and infections. *Ann. Rev. Med.* **26,** 485.

SCHENCK, J.R., HARGIE, M.P., BROWN, M.S., EBERT, D.S., YOO, A.L. & MCINTIRE, F.C. (1969) The enchancement of antibody formation by *Escherichia coli* lipopolysaccharide and detoxified derivatives. *J. Immunol.* **102,** 1411.

SIKRI, K.L., FOSTER, C.L., MACHUGH, N. & MARSHALL, R.D. (1981) Localization of Tamm–Horsfall glycoprotein in the human kidney using immunofluorescence and immunoelectron microscopal techniques. *J. Anat.* **132,** 597.

SOLEZ, K. & HEPTINSTALL, R.H. (1978) Intrarenal urinary extravasation with formation of venous polyps containing Tamm–Horsfall protein. *J. Urol.* **119,** 180.

WHANG, H.Y. & NETER, E. (1967) Further studies on effect of endotoxin on antibody response of rabbit to common antigen of *Enterobacteriaceae*. *J. Immunol.* **98,** 948.

ZAGER, R.A., COTRAN, R.S. & HOYER, J.R. (1978) Pathologic localization of Tamm–Horsfall protein in interstitial deposits in renal disease. *Lab. Invest.* **38,** 52.