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article

## Isolation and characterisation of T lymphocytes from the urethra of patients with acute urethritis

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**Objectives:** To investigate local cellular immune responses in patients with acute urethritis.**Methods:** We have established T cell lines from the urethral exudate and examined their phenotype by flow cytometry. As controls, T cell lines were cultured from first pass urine specimens of asymptomatic healthy individuals.**Results:** Using interleukin 2 (IL-2) alone a T cell line was obtained on only one occasion. Following culture with IL-2, and subsequent expansion by a single stimulation with irradiated allogeneic peripheral blood mononuclear cells (PBMC), phytohaemagglutinin (PHA), and IL-2, it was possible to establish T cell lines from 6/6 acute urethritis patients. T cell lines were also obtained from 4/12 controls subjects, but required repetitive rounds of stimulation with mitogen and allogeneic PBMC to produce sufficient cell numbers for analysis. Three of the patient T cell lines were dominated by T cells expressing the  $\gamma\delta$  receptor.**Conclusion:** The  $\gamma\delta$  T cell subset has been associated with immune responses at mucosal surfaces and has the ability to recognise certain bacterial antigens. The  $\gamma\delta$  T cell response may represent an important aspect of the immune response to organisms associated with acute urethritis. (*Sex Transm Inf* 1998;74:279–283)Keywords: T lymphocytes;  $\gamma\delta$  T cell receptor; urethritis

## Introduction

Organisms such as *Chlamydia trachomatis* (CT) or *Mycoplasma* spp (including *M genitalium*) are isolated in 50–60% of men with non-gonococcal urethritis (NGU). In the remainder no organism can be isolated.<sup>1,2</sup> Urethritis can also be reactive in nature, as seen in patients with gastrointestinal infection complicated by reactive arthritis.<sup>3</sup> In addition, some patients develop recurrent or chronic urethritis despite adequate treatment with appropriate antibiotics and in the absence of new exposure to infection.<sup>1</sup> In these cases the urethritis may be maintained by a failure to eradicate the infection, or conceivably it may be “reactive” in nature. The latter hypothesis has been suggested by Horner *et al* who suggested that immune responses to heat shock protein 60 may be important in pathogenesis.<sup>4</sup>

Each of the organisms associated with urethritis requires an appropriate immune response, and the inflammatory infiltrate leading to the clinical symptom of discharge is evidence of that immune response. Although the discharge is dominated by neutrophils, it is known from animal studies that the critical immune response to an organism such as *C trachomatis* is T cell mediated, reflecting the intracellular location of this organism.<sup>5,6</sup> T lymphocytes have also been implicated in the inflammatory complications of CT induced urethritis, such as reactive arthritis.<sup>7–9</sup> However, little is known about the nature of the T cells which are present within the local inflammatory infiltrate in urethritis. This study has adapted techniques developed to examine lymphocytes in other tissue locations to characterise, for the first time, urethral T cell populations from patients with urethritis.

## Methods

## PATIENTS

Ethical permission was obtained to collect urethral samples from anonymous genitourinary clinic attenders at the General Hospital, Birmingham. Control samples were obtained from laboratory personnel who had no history of urethritis.

Patients with acute urethritis (defined as dysuria, discharge, and five or more polymorphonuclear leucocytes per high power field ( $\times 1000$ ) on urethral smears) were recruited. Urethral swabs were taken for Gram stain and cultures for *Neisseria gonorrhoeae* and *C trachomatis*. Culture for the latter was performed by inoculating coverslips on which McCoy tissue culture monolayers (pretreated with 5-iodo-2-deoxyuridine) were growing with vortex mixed medium in which the swab had been transported. The coverslips were centrifuged at  $2500 \times g$  for 1 hour, incubated at  $35^\circ\text{C}$  for 48–72 hours, and then fixed and stained for the presence of *C trachomatis* inclusion bodies by immunofluorescence (Syva Chlamydia Culture confirmation).

Patients were asked to express the first 2–5 ml of their urine directly into 25 ml of phosphate buffered saline (PBS) in a universal container. A cell pellet was obtained by immediate centrifugation and resuspended in complete medium (CM) consisting of RPMI-1640 medium (Gibco BRL, Paisley) supplemented with 5% heat inactivated human AB+ serum, 2 mM L-glutamine + 100 U/ml penicillin + 100 g/ml streptomycin (Sigma), 1% non-essential amino acids (Sigma), 1% HEPES buffer (Sigma), and 1% sodium pyruvate (Sigma).

Non-inflamed control samples were either collected as above or 2–5 ml of first pass urine were collected directly into CM which was

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centrifuged as above. All cell pellets were then cultured in 2 ml flat bottomed wells overnight in CM containing 50 U/ml interleukin 2 (IL-2) (Chiron UK Ltd, Harefield).

Patient samples were subsequently either cultured in CM containing 50 U IL-2 alone, adding fresh IL-2 containing medium every 3–4 days, or stimulated by co-culturing with  $10^6$ /ml irradiated (3000 rad) allogeneic peripheral blood mononuclear cells (PBMC), 1  $\mu$ g/ml of the T cell mitogen phytohaemagglutinin (PHA, Murex Diagnostics, Dartford, Kent), and 50 U/ml IL-2. Control wells containing irradiated PBMC, PHA, and IL-2 were always cultured in parallel to ensure that outgrowth of inadequately irradiated stimulator cells did not occur. Samples from all 12 controls without clinical urethritis were co-cultured with irradiated PBMC, PHA, and IL-2.

#### CHARACTERISATION OF T CELLS

The surface phenotype of all urethral derived T cell lines was assessed by flow cytometry using an EPICS XL benchtop flow cytometer (Coulter Electronics Ltd, Luton). Aliquots of  $10^5$  cells were stained with directly conjugated fluorescent mouse anti-human monoclonal IgG1 antibodies to the following cell markers: CD3 (UCHT1, Dako Ltd, High Wycombe), CD4 (Leu3A, Becton Dickinson Ltd, Oxford), CD8 (RFT8g, obtained from Dr A Akbar, Royal Free Hospital, London),  $\alpha\beta$  TCR ( $\alpha\beta$  T cell receptor, Becton Dickinson),  $\gamma\delta$  TCR (Becton Dickinson), CD14 (Becton Dickinson), and CD16 (Dako). A fluorescein conjugated mouse IgG1 antibody (Dako) was used as a control.

#### ANTIGENS

Purified protein derivative (PPD) of *Mycobacterium tuberculosis* (Statens Seruminstitut, Copenhagen) was used at a concentration of 10  $\mu$ g/ml.

*C. trachomatis* strain DK20 (oculogenital serovar E) was grown in McCoy cells which were sonicated to release chlamydial elementary bodies. These were purified by centrifugation over Urografin (23 700 g for 2 hours), and used at a concentration of  $1 \times 10^6$  organisms/ml. Sonicated uninfected McCoy cells were used as another control.

*N. gonorrhoeae* was kindly supplied by Alan Jackson, Department of Microbiology, Birmingham Heartlands Hospital, and used at a concentration of  $1.25 \times 10^6$  organisms/ml.

#### PROLIFERATION ASSAYS

PBMC depleted of T cells were used as antigen presenting/accessory cells (APC) as follows: aliquots of  $2 \times 10^5$  cells were cultured in 96 U bottomed plates overnight (16 hours) to allow adherence of APC (macrophages). The wells were thoroughly washed with CM twice to remove the non-adherent cells (mainly T lymphocytes), and  $2 \times 10^4$  T cells in 100  $\mu$ l and the appropriate antigens were then added to a final volume of 200  $\mu$ l in CM. Assays were cultured for 3 days at 37°C in an atmosphere of 5% carbon dioxide, adding 0.15  $\mu$ Ci tritiated thymidine (Amersham, Aylesbury) to each well for

Table 1 Patient characteristics

Patient	Age (years)	CT culture	NG culture	First episode	Notes
A	31	–	–	no	ReA*; NG†
B	25	–	+	yes	
C	35	–	–	no	NG†
D	21	–	+	yes	
E	27	+	–	yes	
F	NA	+	–	yes	

\*Two episodes of reactive arthritis.

†Previous episode of *Neisseria gonorrhoeae* culture positive urethritis.

the final 16 hours of culture. The cells were then harvested and thymidine incorporation measured using a liquid scintillation counter. Results were recorded as mean counts per minute (cpm) (SE).

## Results

#### PATIENTS

The patient characteristics are shown in table 1. Four presented with urethritis for the first time, of which two were culture positive for *C. trachomatis* and two positive for *N. gonorrhoeae*. The other two patients had a previous history of urethritis; patient A had one episode of infection with *N. gonorrhoeae* followed by several additional culture negative episodes, two of which were complicated by the development of arthritis. Patient C had multiple episodes of urethritis, all culture negative for chlamydia, and one episode when *N. gonorrhoeae* was cultured.

#### URETHRAL CELL CULTURE

In initial experiments examination of the urethral cell pellet showed insufficient numbers of lymphocytes for analysis by flow cytometry, so this was only subsequently performed on established T cell lines. Instead all of the cellular contents were cultured in medium and IL-2, and where initial cell numbers permitted, removal of granulocytes and epithelial cells was attempted by centrifugation over Ficoll. Although variable numbers of viable cells with lymphocytic morphology were seen in these cultures, no expansion was observed with the exception of patient A in which substantial numbers of lymphocytes were evident in the cell pellet. In contrast, cell lines were successfully obtained from all six of the patient samples co-cultured with irradiated PBMC, PHA, and IL-2 after a single round of stimulation. No outgrowth occurred in cultures of irradiated PBMC alone examined for more than 4 weeks.

Four of 12 samples from controls without clinical urethritis also yielded cell lines. However, these lines grew much more slowly than those of patient origin, and required two to three rounds of stimulation with PBMC, PHA, and IL-2 to produce sufficient cells for analysis by flow cytometry.

#### FLOW CYTOMETRY

The phenotypes of the urethral derived T cell lines were determined by flow cytometry. The data obtained from the patients with acute urethritis and controls are shown in table 2. In three cases (A, B, F) the T cell line was

Table 2 Phenotypes of T cell lines in patients and controls

	$\alpha\beta+$	$\gamma\delta+$	CD3+	CD4+	CD8+	CD16+	CD14+
Patient A	5%	96%	100%	6%	27%	1%	0%
Patient B	46%	50%	100%	43%	21%	0%	n/d
Patient C	85%	6%	96%	74%	18%	0%	n/d
Patient D	88%	8%	99%	91%	1%	0%	n/d
Patient E	83%	2%	99%	8%	87%	0%	n/d
Patient F	1%	49%	99%	0%	29%	0%	0%
Control 1	98%	0%	100%	98%	6%	0%	0%
Control 2	97%	2%	100%	2%	98%	1%	0%
Control 3	96%	1%	99%	12%	89%	1%	0%
Control 4	96%	4%	99%	99%	3%	3%	0%

dominated by T cells expressing the  $\gamma\delta$  T cell receptor (TCR). In the case of patient F the figure of 49% for TCR  $\gamma\delta+$  cells is falsely low, reflecting relatively weak staining in this particular analysis. Since all the cells were CD3+ and ~1% expressed the  $\alpha\beta$  TCR, it is likely that all the rest were  $\gamma\delta$  TCR positive since CD3 cannot be expressed in the absence of either  $\alpha\beta$  or  $\gamma\delta$  dimers. In two other cases CD4+, TCR  $\alpha\beta+$  cells were in the majority while one case showed mainly CD8+, TCR  $\alpha\beta+$  cells. The urethral T cell lines were phenotypically stable for several weeks in culture, and three rounds of restimulations failed to alter the relative proportions of lymphocyte subsets when reanalysed by flow cytometry (not shown).

For the controls from which urethral T cell lines could be established,  $\gamma\delta$  TCR+ T cells were notable by their absence, and the lines were dominated by either CD4+ or CD8+  $\alpha\beta$  TCR+ cells. Given the small number of lymphocytes in the starting material from these controls, the dominance by either CD4+ or CD8+ cells may represent stochastic expansion of single cells capable of responding to stimulation in vitro.

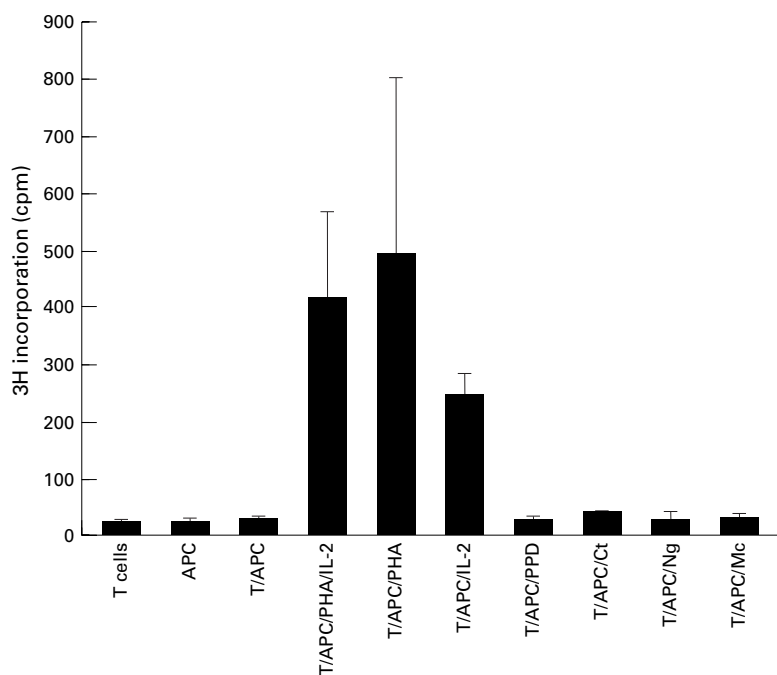


Figure 1 Proliferative responses ( $^3\text{H}$ -thymidine incorporation) of the T cell line from patient A when cultured with irradiated allogeneic antigen presenting cells (APC) either alone or in the presence of antigens, PHA, or IL-2. Ct=Chlamydia trachomatis; Ng=Neisseria gonorrhoeae; Mc= McCoy cells; all as described in Methods. Proliferation of T cells and APC cultured alone is also shown.

#### PROLIFERATION ASSAYS

Preliminary assays were carried out on the urethral T cell line from patient A, which was shown to contain 96%  $\gamma\delta+$  T cells. Although this patient was culture negative for chlamydia on this occasion, and culture positive for gonococcus in the past, the occurrence of reactive arthritis raised the possibility of chlamydial infection not detected by culture. We wished therefore to test whether the urethra derived T cell line was able to respond to chlamydial or gonococcal antigens. Autologous antigen presenting cells were not available from this patient, but in view of the fact that  $\gamma\delta$  cells have not generally been found to show classic MHC restriction<sup>10</sup> allogeneic cells were substituted. As shown in figure 1, the T cell line did not show significant proliferative responses to chlamydial or gonococcal antigens, or to the control antigen PPD which is commonly recognised by  $\gamma\delta$  TCR+ T cells.<sup>11</sup> However, the T cells were capable of mounting a satisfactory response to PHA and IL-2.

#### Discussion

In this report, we have established, for the first time, the feasibility of deriving polyclonal T lymphocyte cell lines from urethral exudate in patients with active urethritis. Furthermore, we have shown that material from the healthy urethra does not give rise to T cell lines with the same frequency or in the same time frame, but some T lymphocytes are clearly present in the material, since T cell lines appear from a minority of specimens after prolonged culture and multiple rounds of stimulation. An earlier study from this centre described the enumeration of CD2+ and CD4+ T cells in first void urine of urethritis patients using antibody coated magnetic beads,<sup>12</sup> but the ability to culture the cells opens the way for more detailed study of the phenotype and function of urethral T cells in the pathogenesis of urethritis.

To what extent do the properties of the T cell lines isolated in vitro mirror those of the initial population present in the urethra? The methodology used—that is, stimulation with mitogen and accessory cells, has the ability to expand all T cell subsets, and therefore is not in itself selective. Likewise, we cultured all those T cells present in the cell pellet obtained by centrifugation, a less selective procedure than isolation over Ficoll, and a method which includes T cells which were adherent to mucin in the discharge material. However, different T cell subsets may well have differing viabilities on being shed into the urethra, and only those cells capable of responding to stimulation by cell division will contribute to the T cell line. In this preliminary study we did not assess the reproducibility as regards the phenotype of the cell lines obtained—for example, by splitting the discharge into replicate cultures, but this should be examined in future studies. Where small numbers of T cells are present initially, as is the case in controls, it is possible that the phenotype of the T cell lines obtained will be determined stochastically, each line representing expansion of a small number of T cells present. Where larger cell numbers are present



as part of an inflammatory infiltrate, a consistent phenotype in the T cell lines obtained would be expected.

Although this initial pilot study investigated a group of patients which was heterogeneous with respect to clinical history and infecting organisms, some interesting findings were recorded. Firstly, 3/6 of the T cell lines contained a large proportion of cells expressing the  $\gamma\delta$  TCR, whereas these cells were not evident in the small number of lines obtained from controls. Only a small proportion of the peripheral blood T cell population is made up of  $\gamma\delta$  T cells, the rest being  $\alpha\beta$  T cells.<sup>13</sup>  $\gamma\delta$  T cells have been shown to have a role both in mucosal immunity, though mainly in murine systems,<sup>11</sup> and in response to infection by intracellular pathogens.<sup>14 15</sup> Although experiments with rodents in which the TCR  $\delta$  gene has been inactivated ( $\gamma\delta$  TCR "knockout" mice) have suggested that this subset is not critical to eventual recovery from infection with mycobacteria or *C trachomatis*,<sup>16</sup> the subset may still have a role in the initial local response to infection.<sup>17</sup>  $\gamma\delta$  T cells are resident in epithelial tissues including the genital tract, and in the mouse,  $\gamma\delta$  cells with particular invariant receptors are seen in both the gut and vagina, although a recent report commented on their relative absence from the murine male urethra.<sup>18</sup> In humans  $\gamma\delta$  cells are also commonly found in gut mucosa in both intraepithelial and submucosal situations,<sup>19</sup> but their precise role in mucosal immunity has not been defined. They recognise non-protein antigens, particularly certain bacterial products, which do not require either the processing mechanisms or the MHC restriction elements used by protein antigens.<sup>20-22</sup> It has also been suggested that  $\gamma\delta$  T cell antigen recognition may be more similar to recognition of antigen by immunoglobulin.<sup>23</sup> Together these features may mean that  $\gamma\delta$  T cells act early in response to infection, possibly as part of the innate immune system.<sup>17</sup>

In view of the small numbers of patients examined no clear correlation can be made between the phenotype of the urethral T cell lines obtained and the clinical features. Infection with *C trachomatis* was only established by culture of the organism which has a significant proportion of false negatives. In future studies it will be important to establish as accurately as possible the nature of any infecting organism, using techniques such as polymerase or ligase chain reaction. Ideally these tests should also be performed on control urethral specimens; in the current pilot study where laboratory volunteers were used this was not thought to be appropriate. However, it is possible that the minority of specimens from which T cell lines were eventually established were those with a low grade subclinical urethritis. In addition to the nature of the organism triggering the acute urethritis, factors such as duration of symptoms and previous exposure to urethritis inducing organisms (including subclinical exposure) may all influence the phenotype of T cells found in the urethra. These aspects also require future study.

Preliminary experiments designed to determine whether the urethral T cell line composed almost wholly of  $\gamma\delta$  T cells could recognise bacterial antigens did not show specificity. It is possible that the allogeneic antigen presenting cells did not have the requisite restricting element; as noted above classic MHC antigens are not usually required by  $\gamma\delta$  T cells, but some see antigen in the context of non-classic MHC antigens such as CDI.<sup>24</sup> This may not have been adequately expressed by the antigen presenting cells used in our assay. Alternatively, the stimulatory antigen may not have been released by processing from the whole organism preparations which we used to stimulate. Future studies may use cloning and sequencing of the T cell receptors used by urethral  $\gamma\delta$  T cells to determine if they display common features or features similar to peripheral blood  $\gamma\delta$  T cells stimulated with bacterial antigens. We have recently illustrated the feasibility of this approach with respect to the  $\gamma\delta$  T cell response to *Yersinia enterocolitica* and *Campylobacter jejuni*.<sup>25 26</sup> A previous study noted the relatively large proportion of  $\gamma\delta$  T cells in semen, and showed that there were larger numbers in patients with immune responses to sperm.<sup>27</sup> Culturing peripheral blood T cells from these patients with autologous sperm resulted in expansion of the  $\gamma\delta$  T cells, suggesting that they could recognise spermatozoal antigens.<sup>28</sup> Whether such cells account for some of those isolated from urethra in our study, and whether  $\gamma\delta$  T cells stimulated by infective agents might cross react with spermatozoal antigens is another possibility which should be pursued.

*Contributors:* The laboratory work was carried out by Ms Brunst under supervision of Drs Gaston and Pearce; Dr Sukthankar obtained urethral samples and documented clinical details under the supervision of Dr Shahmanesh. The project was designed and initiated by Drs Gaston and Shahmanesh and the manuscript was written collaboratively by Ms Brunst, Dr Gaston, and Dr Shahmanesh.

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