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CHAPTER 10

ANTIGEN PRESENTATION AS A FACTOR IN IMMUNITY TO RENAL INFECTION
INTRODUCTION

Urinary tract infections are commonly encountered in general medical practice and although they usually respond to antibiotic therapy, they frequently reoccur and in many cases are difficult to eradicate. As a result, a number of investigators have considered utilizing the immune response of the host as a protective measure in an attempt to prevent this pattern of infection from becoming established.

Several studies in experimental animals have shown promising results (Kaijser & Olling, 1973; Sanford, Hunter & Souda, 1962; Brooks, Lyons & Braude, 1974) while others have not been able to demonstrate any protective value (Montgomerie et al., 1972; Uehling & Constant, 1970) so that the question remains unresolved. A reason for this discrepancy may be the differing susceptibility to infection of the animal species used, but variations in the primary immunizing stimulus is an alternative and more likely explanation.

Although there has been considerable interest in immunization as a protective measure, there has been no systematic investigation of the effect of the initial antigenic stimulus on the secondary response to challenge. In the current experiments the effect of varying amounts and different methods of presentation of the immunizing antigen on the host protective secondary immune response has been investigated.

MATERIALS AND METHODS

Animals
Female animals weighing 215 to 225 g. were obtained from a random bred strain of Wistar rat.

Antigen stimuli
Killed bacterial antigen was prepared by growing *E. coli* 075 in nutrient broth to a concentration of $1 \times 10^9$ organisms per ml. The culture was steamed for two hours and washed twice in phosphate buffered saline (PBS) and resuspended in its original volume. Groups of animals were injected intramuscularly with ten-fold increments of antigen commencing with $1 \times 10^6$ killed bacteria. Immunization with live bacteria was carried out by injecting the animals intravenously with 1 ml. of a washed culture of *E. coli* 075 containing $1 \times 10^6$ to $1 \times 10^9$ viable bacteria. In the case of active infection, pyelonephritis was induced by the direct inoculation of $1 \times 10^3$ to $1 \times 10^6$ viable bacteria into the exposed kidney. Details of this method have been given in Chapter 2.

Blood samples
Blood was obtained from the tip of the rat's tail and collected directly into a 0.5 ml. microfuge tube. After separation, serum samples were stored at $-20^\circ$C until analyzed.

Bacteriological examination of blood, tissue and urine
Quantitative bacterial counts were carried out on tissues and blood after the intravenous injection of viable organisms and after the direct inoculation of bacteria into the kidney. Groups of animals were killed at intervals and the tissues removed under sterile conditions before being homogenized in a Tri-R tissue homogenizer (Rockville Centre, New York). Nutrient agar pour plates of serial ten-fold dilutions of
kidney homogenate were made to obtain the bacterial count per gram of wet tissue. Blood samples were obtained by heart puncture and diluted in broth before being inoculated onto the surface of a blood agar plate. Urine samples obtained by bladder puncture at autopsy were treated in a similar manner.

**Determination of serum antibody responses**

Antibody levels were determined by a passive haemagglutination procedure using semi-automated microtitre apparatus fitted with 50 μl diluters (Cooke Microtitre Minidiluter). Boivin type endotoxin for the sensitization of the sheep-red-blood-cells (SRBC) was prepared by trichoracetic acid extraction of a culture of *E. coli* 075 essentially as described by Webster et al. (1955).

Activation of the endotoxin preparation was carried out by heating the endotoxin in an alkaline medium. A solution of endotoxin, 0.12 mg. per ml., was prepared in 0.02N NaOH and heated for five minutes in a boiling waterbath. On cooling the pH was adjusted to 7, and an equal volume of 0.3M PBS was added. This preparation was stable and maintained its activity after several weeks' storage at 4°C. To sensitize the SRBC, equal volumes of washed SRBC and activated endotoxin were mixed and incubated at 37°C for two hours, then washed three times with PBS. Sensitization of SRBC was confirmed by carrying out passive haemagglutination tests with an anti-*E. coli* 075 serum that had been standardized against a bacterial antigen.

All dilutions of the sera were prepared in duplicate. SRBC sensitized with bacterial antigen were added to one set of dilutions and an equal volume of antigen containing
2-mercaptoethanol (2ME) in a final concentration of 0.1 M was added to the second set of sera. The plates were sealed and incubated for one hour at 37°C and held overnight at 4°C. Titres were expressed as the reciprocal of the highest dilution showing gross agglutination. The validity of this method for determining the total antibody and the proportion of antibody that is resistant to 2ME (IgG) was confirmed by comparing the results obtained by the present method with the results of titrations of antibody after serum samples had been treated with 2ME.

Production of T cell depleted experimental animals by thymectomy and sublethal irradiation (TXIR)

See Chapter 9 for details.

In vitro assay of phytohaemagglutinin (PHA) stimulated mitogenesis of thymus derived lymphocytes

Lymphoid cells were obtained by removing the spleen under sterile conditions into ice-cold HEPES-buffered Eagles minimum-essential-medium (HEPES-MEM, Grand Island Biological Co., New York). Each spleen was gently minced with curved iris scissors and the tissue fragments teased through a 60 mesh stainless steel gauze to produce a single cell suspension which was washed once and resuspended in HEPES-MEM. The cell suspension was then counted and sufficient cells sampled to provide a culture in triplicate each containing $5 \times 10^6$ lymphocytes. Full details of the culture method have been given in the previous chapter.
RESULTS

Bacteriology

The distribution of viable bacteria in the spleen, liver, kidney and blood was determined following the intravenous injection of $1 \times 10^8$ E. coli 075 and the inoculation of $1 \times 10^5$ bacteria directly into the kidney (Figure 58).

Seventy-two hours after injection, the number of bacteria in the spleen, liver and kidney were reduced to 0.16, 0.0001 and 0.25% respectively, of the number present immediately after challenge. In contrast, direct inoculation of E. coli 075 into the kidney resulted in bacterial replication and the number present 48 hours after challenge increased from $1 \times 10^5$ to $3.9 \times 10^7$ per gram. In a further experiment, increasing numbers of viable bacteria from $3 \times 10^1$ to $3 \times 10^5$ were injected directly into the kidney and the number of viable organisms present in the kidney three days later was determined. There were no significant differences in the number of viable bacteria in infected kidneys three days after the direct inoculation (Figure 59) of $3 \times 10^2$ to $3 \times 10^5$ organisms into the kidney.

When the gross and histopathologic changes in the various groups were compared at autopsy, similar results were found in all groups so that the pathological and bacteriological features three days after challenge were independent of the number of organisms used to initiate infection.

Effect of primary immunization on the secondary immune response

Killed antigen: four groups of animals, each group containing
FIGURE 58.

Distribution of bacteria in the blood, liver, spleen and kidney after the intravenous injection of $1 \times 10^8$ viable E.coli 075 and the direct inoculation of $1 \times 10^5$ viable E.coli into the kidney. Each point is the mean of the results from four animals.
I, to J. Et-cDLo-o-o-o-ra-o-Lo-cttJ.

1.8 262t, Hours after injection

Log no. of bacteria per gram

8
7
6
direct inoculation
kidney

5
spleen

4

3
kidney
I.V. inoculation

2

1

blood

0 2 6 24 48 72

Hours after injection
FIGURE 59.

Bacterial numbers in the kidney and urine 3 days after the direct inoculation of varying numbers of *E.coli* 075 into the kidney.
eight to ten rats were immunized with increasing amounts of killed bacterial antigen injected intramuscularly and containing $1 \times 10^6$ to $1 \times 10^9$ heat killed organisms. Thirty-five days after the primary immunization, all animals were challenged with a standard secondary challenge of $1 \times 10^9$ heat-killed bacteria. Serum antibody levels were determined after primary immunization and following the secondary challenge. The results are shown in Figure 60. Groups of animals challenged with the three lower doses of killed antigen all showed a similar response to immunization but the response to the maximum dose of killed antigen was considerably higher than the lower doses. An occasional animal showed a feeble response to primary immunization and was not included in the calculation of the mean titre. All animals showed a greatly enhanced secondary response to a standard challenge irrespective of the immunizing dose. In contrast to the primary immunization where an increasing serum antibody was barely discernible on the third day, a greatly enhanced antibody titre was consistently found after a secondary challenge. The peak titre was again found seven days after challenge and all groups showed an increase in maximum serum antibody levels when the secondary response to $1 \times 10^9$ killed organisms was compared with the primary response to the same challenge. The production of specific antibacterial IgG antibody was of particular interest and although negligible amounts were produced on primary immunization considerable titres were found on secondary challenge. In individual animals, the titre of the IgG antibody was equal to the total antibody titre and IgG antibody levels of up to 25% of the total antibody were commonly found. An
FIGURE 60.

Effect of primary immunization with $1 \times 10^6$ to $1 \times 10^9$ heat killed organisms (*E. coli* 075), injected intramuscularly on the secondary immune response. The secondary challenge of $1 \times 10^9$ heat killed organisms was standard for all four groups. The total antibody titre is represented by the unshaded area and that part of the total antibody which was IgG, by the cross hatching.
inverse relationship between the immunizing dose and the level of IgG antibody was also observed and the highest titres of IgG antibody were found in animals primed with the lower doses of antigen.

Systemically administered live antigen: immunizing doses of $1 \times 10^6$ to $1 \times 10^9$ live bacteria were injected intravenously into four groups of ten animals. Thirty-five days after the immunizing stimulus, all groups were challenged with $1 \times 10^9$ heat killed bacteria. Serum antibody levels were shown in Figure 61. A slight but definite rise in antibody was found three days after primary immunization with the live antigen but peak titres were again found seven days after challenge. Antibody titres were generally higher with the greater immunizing doses, but the titre of IgG antibody remained low in all groups.

When the animals were rechallenged with a standard dose of killed organisms, a marked response was seen three days after the secondary challenge and increased levels of IgG antibody were also found. Peak serum antibody titres on secondary challenge were higher than those following primary immunization with the same amount of killed antigen but the differences between immunization with viable organisms and the secondary challenge with killed antigen were less marked than the previous experiment when killed antigen was used as the primary immunization as well as the secondary challenge.

Active renal infection in immunization: pyelonephritis was produced in four groups of ten animals. Bacteria were introduced directly into the kidney in ten-fold increments
FIGURE 61.

Effect of primary immunization with $1 \times 10^6$ to $1 \times 10^9$ viable organisms (E.coli 075), injected intravenously, on the secondary immune response. The secondary challenge of $1 \times 10^9$ heat killed organisms was standard for all four groups. The total antibody titre is represented by the unshaded area and that part of the total antibody which was IgG, by the cross hatching.
commencing with $1 \times 10^3$ viable organisms in the first group. Replication of bacteria occurred in the directly infected kidney in contrast to the steady clearance from liver, lung, spleen, blood and kidney when the organisms were injected intravenously.

Bacterial proliferation in the kidney did affect both the primary and secondary immune response (Figure 62). In contrast to the other two groups immunized with killed and viable non-replicating antigen, an increase in serum antibody was found three days after renal infection with the exception of the lowest challenge dose. Peak antibody titres were again found seven days after challenge and were several-fold higher than those in the other antigen forms but the peaks were not related to the amount of the challenging dose. The levels of IgG antibody after immunization with active renal infection were also considerably higher than when live or killed antigen was used as an immunizing agent.

A further secondary challenge with killed antigen did not result in a secondary response as in the previous experiments where animals had been primed with killed bacterial antigen or viable bacteria injected intravenously. Although an increase in serum antibody titres was seen, in some groups the peak titre of the secondary challenge was less than the peak titre found during the primary response. The IgG antibody titre varied considerably but secondary stimulation did not result in a consistently increased IgG serum antibody level.

**Duration of immunological memory**

Thirty rats were immunized with $1 \times 10^7$ killed organisms and
FIGURE 62.

Effect of active renal infection induced by the direct inoculation into the kidney of varying numbers of *E. coli* 075, from $1 \times 10^3$ to $1 \times 10^6$, on the secondary immune response. The secondary challenge of $1 \times 10^9$ heat killed organisms was standard for all four groups. The total antibody titre is represented by the unshaded area and that part of the total antibody which was IgG, by the cross hatching.
divided into three equal groups. One group was rechallenged with $1 \times 10^9$ heat killed organisms one month after immunization and the second and third groups left four and six months before being similarly rechallenged. Serum antibody titres in both groups were determined and compared with control groups which had been caged under the same conditions as the test group but had not been previously immunized. Immunological memory was readily demonstrable one month after immunization and confirmed the earlier results (Figure 63). When the secondary immune response of animals immunized four and six months previously was determined, a greatly enhanced secondary response was again found when the immune response of these animals was compared with non-immunized controls.

**Immunological memory in animals lacking T lymphocytes**

These experiments were carried out to determine the type of lymphocyte which carries immunological memory to bacterial antigen. Nine animals were thymectomized and submitted to serial sublethal irradiation to produce rats lacking functional T lymphocytes (TXIR), nine were irradiated only (IR) and a further nine served as controls. Three weeks after the final irradiation, all animals were given an immunizing dose of $1 \times 10^7$ killed organisms and again challenged 30 days later with $1 \times 10^9$ killed organisms to evaluate the effect of T cell ablation on immunologic memory. A typical secondary immune response with enhanced antibody production was found three days after secondary challenge and increased amounts of IgG antibody was seen in all three groups (Figure 64). Lack of functional T cells did not affect the development of immunologic memory in thymectomized and T cell ablated animals.
FIGURE 63.

Duration of immunological memory. Three groups of animals were immunized with $1 \times 10^7$ killed organisms (E. coli 075) and a secondary challenge with $1 \times 10^9$ killed organisms carried out with individual groups 1, 4 and 6 months later. The secondary immune response of each group was compared with the immune response of a non-immunized group. The total antibody titre is represented by the unshaded area and that part of the total antibody which was IgG, by the shaded area.
IMMUNOLOGIC MEMORY

Non-immunized (control) | 1 month | Immunized

Non-immunized (control) | 4 month | Immunized

Non-immunized (control) | 6 month | Immunized
FIGURE 64.

Effect of T cell ablation on the carriage of immunological memory. Three groups of animals were immunized with $1 \times 10^7$ killed organisms after the T cell function had been ablated in one group by thymectomy and irradiation (TXIR). The other two groups, irradiated only (IR) and non-manipulated animals, served as controls. Thirty days later, all animals were challenged with $1 \times 10^9$ killed organisms. The total antibody titres following the secondary challenge are shown by the unshaded area and that part of the total antibody which was IgG, by the shaded area.
T CELL INDEPENDENT MEMORY

Control
1° response

Control
2° response

SERUM ANTIBODY TITER

DAYS AFTER CHALLENGE

TXIR
2° response

IR
2° response
At the completion of the experiments, the rats were sacrificed and the functional capacity of the T lymphocytes in the three groups was quantitated by determining the PHA responsiveness of the splenocytes in tissue culture. The mean PHA response of the eight control animals was 49,830 D.P.M./10^6 lymphocytes while the comparable response for the T cell ablated group was 1447 D.P.M. and represented a 97% ablation of T cell function in the TXIR group.

DISCUSSION

Antibody to the somatic antigen of E.coli may be an important component of the immune defence mechanism involved in protection against recurrent renal infection. Bacterial lipopolysaccharide in particular persists for extended periods (Aoki et al., 1967; Cotran, 1963; Sanford, Hunter & Donaldson, 1962; Britton, Wepsic & Moller, 1968) and this characteristic could affect the ability of the host to respond to a second challenge. Many investigators have noted the marked effect of antigen dose on the antibody response to a variety of Gram negative organisms (Pike & Schulze, 1964; Gowland, 1974; Gras et al., 1974; Landy, Sanderson & Jackson, 1965) and it is widely appreciated that the magnitude of the secondary immune response is dependent on the nature of the primary immunization (Blazkovec & Wolfe, 1965; Nakashima et al., 1974; Makela & Mitchison, 1965).

In these experiments, it was shown that a considerable variation in the dose of killed antigen used as a primary immunizing agent did not affect the secondary immune response to any degree. In all groups, a characteristic secondary immune
response to a standard challenge was found and an increase in serum antibody levels was observed earlier than during the primary immune response. An increase in the titre of IgG antibody was also found and this was inversely proportional to the amount of antigen used in the primary immunization. When a similar number of live organisms was used as an immunizing antigen, a typical secondary immune response was found which again did not seem to be affected by the nature of the primary immunization.

Replication of viable bacteria in the kidney, however, did have a noticeable effect on the titre of serum antibody during the primary immune response. Peak serum antibody titres were approximately ten-fold higher following renal infection than when live antigen was injected intravenously, even though the challenging inoculum was 1000-fold less in the case of renal infection. In renal infection too, the secondary challenge failed to increase the serum antibody titre beyond the peak found during the primary response although previous infection did result in considerably greater concentrations of antibody early in the secondary response.

The main concern in these experiments was to determine the most appropriate form of antigen presentation for the stimulation of protective antibody in renal infections or alternatively to disclose immunization schedules that would prevent a less than optimum secondary immune response.

The assessment of the effect of active renal infection itself on the subsequent immune capabilities of the host was of particular importance. In practice, this is probably the most
common form of antigenic challenge presented to the host and from an immunologic viewpoint, the chronicity of many renal infections presents additional problems as there is no lapse in antigenic stimulation between the primary challenge and any subsequent activation of the infection. In these experiments a wide range of antigenic stimuli including live and killed antigen as well as active renal infection was used as the immunizing stimuli. The ability of the host to respond to a further challenge was unimpaired, however, and suggests that some of the factors limiting the secondary immune response to several other antigens may not be so important in the case of the somatic antigens of E.coli.

If it is accepted for argument's sake that the humoral immune response to bacterial antigen may have a protective effect in renal infection, there are still a number of obstacles to be overcome to determine the optimum conditions for immunization. The characteristics of the protective immunoglobulin, for instance, have not been determined although Ahlstedt believes that the protective capacity is related to the titre of specific antibody and that antibody class and avidity may be important (Ahlstedt, Holmgren & Hanson, 1973).

The observation that most cases of recurrent renal infection are due to reinfection with a different seriological strain of E.coli (Bergstrom et al., 1967; Gruneberg, 1970) would also limit the effectiveness of a specific immune response and could explain the lack of convincing clinical evidence for protective humoral immunity. The experiments of Holmgren and Ahlstedt are of interest in this respect and suggest that prior exposure
to serologically unrelated E. coli enhances the capacity of the host to form IgG antibody but that the IgM response may be suppressed (Holmgren & Ahlstedt, 1974). Attempts to immunize animals against heterogenous Gram negative bacilli using the common antigen of E. coli as an immunizing agent have not been successful (McCabe & Greely, 1973) and underscore the difficulties in providing adequate immunity against the range of E. coli serotypes encountered in renal infection. Encouraging results have been obtained, however, in similar experiments where the Re core antigen from lipopolysaccharides of an R mutant of Salmonella minnesota was used as an immunizing agent (McCabe, 1972).

The present experiments have confirmed other reports demonstrating immunological memory to the somatic antigen of E. coli (Altemeier, Robbins & Smith, 1966; Landy, Sanderson & Jackson, 1965; Holmgren, 1970) and have shown that memory persists for at least six months after the primary immunization.

It is now well established that immunological memory is the responsibility of the small lymphocyte (Gowans & Uhr, 1966; Hunt, Ellis & Gowans, 1972; Ellis, Gowans & Howard, 1969) and in the current experiments, it has been shown that memory to the somatic antigen of E. coli is carried by the B lymphocyte. Particular care was taken to assay the residual T cell function in the T cell ablated animals used in these experiments and this was found to be less than 3% of normal. The data show that, despite a lack of functional T cells, immunological memory to bacterial antigen developed normally and a typical secondary response was found when animals were challenged one
month after immunization.

Several authors have demonstrated T memory cells (Rubin & Coons, 1972; Cunningham & Sercarz, 1971; Raff, 1970; Falkoff & Kettman, 1972) but B memory cells have also been found following immunization with several antigens including bacterial antigen (Jacobsen, L'Age-Stehr & Herzenberg, 1970; Roelants & Askonas, 1972; Andersson & Blomgren, 1971; Iverson, 1973). Mond et al. (1974) have raised the question of the possible T cell dependence on an early B cell activation step in the immune response to Br. abortus which would not be affected by transfer experiments. These experiments would seem to have ruled out this possibility, at least for E.coli lipopolysaccharide, as the animals were deprived of T cells at the time of primary immunization.

The present studies, as well as being concerned with the immune response to bacterial lipopolysaccharides, also contribute to an appreciation of the possible role of immunization in the management of renal infection. The experiments have shown that all the characteristics of a secondary immune response can be demonstrated with this bacterial antigen although much of the basic immunobiology of the response remains to be determined.
CHAPTER 11

QUANTITATION OF IMMUNOGLOBULIN-BEARING LYMPHOCYTES
AND THE LYMPHOCYTE RESPONSE TO PHA IN
EXPERIMENTAL PYELONEPHRITIS
INTRODUCTION

One of the characteristic features of pyelonephritis in man and experimental animals has been the presence of a lymphocytic infiltrate in the kidney. This has provided a stimulus to investigate the role of the small lymphocyte in the inflammatory pyelonephritic lesion but much of the basic immunobiology of lymphocyte mobilization to the infected kidney is still unknown. Now that it is possible to divide lymphocytes into the bursal equivalent B cells and the thymus-derived T cells, it has become important to determine the role of B cell mediated humoral immunity and T cell directed cell-mediated immunity on the course of infection and in any resultant immunity. In the present experiments, the movement of T and B cells into the kidney in pyelonephritis has been studied and the changes that occur in lymphoid populations in response to renal infection have been described. The effect of infection on the functional capacity of T cells in peripheral blood in lymphoid tissue as well as within the pyelonephritic kidney was also evaluated.

MATERIALS AND METHODS

Animal strain
Female rats weighing 225 to 250 g., obtained from a random-bred strain of Wistar rat, were used.

Production of renal infection
Pyelonephritis was induced by the direct inoculation of E. coli 075 into the surgically exposed kidney using a glass micro-capillary tube. Details of the method have been given
Identification of bursal equivalent "B" cells

Autoradiography, using a rabbit anti-rat immunoglobulin serum labelled with $^{125}$I was used to identify B cells as lymphocytes carrying surface immunoglobulins. Lymphocytes failing to show surface labelling were considered to be T cells. Rat immunoglobulin was obtained by 35% ammonium sulphate saturation of rat serum. The precipitated globulin fraction was dialysed free of ammonium sulphate and then lyophilized. An antiserum against the immunoglobulin fraction was prepared by the repeated immunization of a rabbit with the protein which was emulsified in Freund's adjuvant for the initial injections. The IgG component of this fraction was isolated by elution from diethylaminoethyl cellulose (DEAE) with 0.01M buffer at pH 7.0.

Iodination of the antiserum

Fifty μg. of the IgG fraction of the antirat immunoglobulin serum were diluted in phosphate buffered saline to give a final volume of 100 μl. $^{125}$I labelling was carried out basically as described by Hunter & Greenwood (1962). 500 μCi. of $^{125}$I (Amersham, specific activity 14 μCi./μg.) and 10 μl. of choline T (1 mg./ml.) were added to the antiserum in a reaction vial and the preparation stirred on ice with a micro-bar magnet for ten minutes. 10 μl. of sodium metabisulphate (2.4 mg./ml.), 20 μl. of bovine serum albumin (50 mg./ml.) and 20 μl. of potassium iodide (15.5 mg./ml.) were then added and the contents of the vial transferred in 1 ml. of potassium iodide solution to a 5 cm. G25 sephadex column. The labelled protein was then eluted using phosphate buffered saline and 0.2 ml.
samples were collected and counted in a gamma well-crystal scintillation counter (Ekco Electronics Ltd., Type M610B) to identify the fraction containing the protein peak. The labelling efficiency determined by paper electrophoresis was found to be 92% in a series of 30 labellings, giving an average specific activity of 9.2 μCi/μg. of protein.

Preparation of cell suspensions
Lymphocyte suspensions were prepared from the spleen, thymus, perirenal nodes, peripheral lymph nodes, pyelonephritic kidney tissue and peripheral blood. Peripheral blood lymphocytes were obtained at the time of sacrifice from 4.5 ml. of blood collected by heart puncture. The blood was collected in a syringe containing 0.5 ml. of 5% EDTA and then added to 15 ml. of chilled Eagle's minimal essential medium buffered with HEPES (HEPES-MEM). The lymphocytes were separated from the diluted blood using a Ficoll-Conray 280 reagent (Douglas & Figgins, 1971). Lymphocytes were obtained from lymphoid tissues by gently teasing the cells through a 60 mesh stainless steel sieve into HEPES-MEM. Lymphocyte suspensions from lymph nodes, spleens and thymus were filtered through a loosely packed plug of cotton wool in the bottom of a 5 cm. column to remove clumps of cells. Wedges of pyelonephritic kidney tissue were also teased into HEPES-MEM and the cell suspension centrifuged at 500 g. through a 50% foetal calf serum-foetal calf serum gradient to remove cellular debris.

Cell labelling and autoradiography
7.5 x 10^6 lymphocytes were added to a 15 ml. conical glass centrifuge tube and the cells deposited by centrifugation at
400 g. for five minutes. 1.5 μg. of labelled antiserum protein was added to the button of cells in each tube and the volume made up to 0.25 ml. with Eagle's MEM. The tubes were held on ice for one hour before resuspending the button in 1 ml. of HEPES-MEM and layering onto a gradient of 50% foetal calf serum and foetal calf serum. The cells were centrifuged through two such gradients to remove unincorporated label and then smeared on gelatin coated slides. After air drying they were fixed in a methanol-acetic-acid-water (80, 10, 10) fixative and finally dipped in NTB-2 liquid photographic emulsion. The slides were stored in light tight boxes at 4°C and developed in Kodak D19 at 17°C. after 5, 7 and 10 days' exposure. Finally they were stained with Leishman's stain and at least 300 lymphocytes examined for the presence of surface labelling with the 125I anti-rat immunoglobulin. In most instances lymphocytes were either heavily labelled or were unlabelled so that differentiation did not present a problem. When less heavily labelled cells were encountered, they were compared with the degree of labelling of a thymocyte population included in each experiment (Figure 65).

**In vitro assay of phytohaemagglutinin (PHA) stimulated mitogenesis of T lymphocytes**

Details have been given in Chapter 9.

**RESULTS**

**Percentage of B and T lymphocytes in representative lymphoid tissues**

Lymphoid suspensions were prepared from peripheral blood and
FIGURE 65.

Auto-radiography, using rabbit anti-rat immunoglobulin serum labelled with $^{125}$I was used to identify B cells as lymphocytes carrying surface immunoglobulin. Lymphocytes failing to show surface labelling were considered to be T cells.
lymphoid tissue from rats in 34 separate experiments and the percentage of lymphocytes carrying surface immunoglobulins (B cells) determined using an anti-immunoglobulin serum labelled with $^{125}$I. The results are shown in Figure 66. Splenic tissue contained the most B lymphocytes (38%), followed by peripheral blood lymphocytes (34%), lymph nodes (30%) and thymus (1%). When the distribution of B lymphocytes in the spleen was determined in ten individual rats in a single experiment, a mean of $44 \pm 4.3$ was found which gave a coefficient of variation of 10% and similar variations were found with lymph node suspensions and peripheral blood lymphocytes. In subsequent experiments, the range of values obtained with normal animals was considered to represent technical rather than biological variations and in each experiment, two animals were sacrificed, a pyelonephritic animal and a normal control.

Distribution of B lymphocytes in renal infection
Unilateral pyelonephritis was induced in a total of 22 animals. Experiments were carried out 3, 7, 14 and 28 days after challenge to determine the effect of acute, resolving and healed pyelonephritis on the distribution of B cells in lymphoid tissue. At sacrifice, lymphocyte suspensions were prepared from perirenal nodes, peripheral lymph nodes distant to the site of infection (lumbar, cervical, axillary) peripheral blood and the infected region of the pyelonephritic kidney. In each experiment lymphocyte suspensions were prepared from pyelonephritic and normal animals and the proportion of labelled lymphocytes from each lymphoid site in pyelonephritic animals were expressed as a percentage of the labelled cells
FIGURE 66.

Percentage of T and B lymphocytes in the spleen, peripheral blood (PBL), lymph nodes distant to the infection (DLN) and the thymus of the rat strain used in these experiments.
found in the comparable tissue from the normal animal (Figure 67).

Three days after the challenge, there was a reduction in the percentage of B cells in the spleen, but by seven days, the percentage of B cells in the spleen had increased. Fourteen days after infection, three of the four animals showed a further drop in the percentage of B cells, although this result was reversed during resolution of the lesion 21 and 28 days later. In the case of the peripheral blood lymphocytes, a consistent increase in the percentage of B cells was found three and seven days after infection, but thereafter no regular pattern persisted. An increase in the number of B lymphocytes in the peri-renal node was found early in infection, but this finding was again reversed during the 14 to 28 day period. There were no consistent changes in the proportion of lymphocytes in lymph nodes distant from the site of infection.

**Lymphocytic infiltrate in pyelonephritis**

Surface immunoglobulins could not be demonstrated on the majority of lymphocytes forming the lymphocytic infiltrate in the pyelonephritic kidney and these cells have been presumptively identified as T lymphocytes (Figure 68). B lymphocytes labelled satisfactorily with the anti-immunoglobulin serum and there was little difficulty distinguishing renal epithelial cells from the lymphocytes on morphological grounds. No labelling of the renal epithelial cells in the preparation was found.
FIGURE 67.

The effect of renal infection on the distribution of B lymphocytes in the spleen, lymph nodes distant to the infection (DLN), peripheral blood lymphocytes (PBL) at intervals after the induction of pyelonephritis. The stippled area represents the biological variation (coefficient of variation) in the percentage of B cells.
FIGURE 68.

Percentage of T and B lymphocytes in the lymphocytic infiltrate of the pyelonephritic kidney during the course of infection.
Lymphocytic infiltrate
% T and B cells

KIDNEY

Days after renal infection

T  B  T  B  T  B  T  B
7  14  21  28

= T cells
= B cells
Functional capacity of T lymphocytes during the course of renal infection

Thymus derived lymphocytes in culture respond to stimulation with PHA by blastogenesis and an increase in DNA synthesis. In these experiments, the ability of lymphocytes from various lymphoid sites and the kidney to respond to PHA stimulation was used as an index of their functional capacity in pyelonephritis. In 41 consecutive cultures of splenocytes from normal animals, an average of 58,000 (SD 18,000) DPM/10^6 lymphocytes was found. The same system of internal controls used to assess the B lymphocyte labelling data was used in these experiments. With each experiment, two age-matched normal animals were sacrificed, similar lymphoid tissues pooled, and cultures prepared in parallel with the test animal. The results (Figure 69) are expressed as a percentage of the response of lymphocytes from normal animals. A comparison of the PHA responsiveness of lymphoid tissues within the infected animals was also made. A marked decrease in the PHA responsiveness of T lymphocytes in the spleen was found in all the animals studied three days after infection. Recovery of PHA responsiveness occurred with time, but some depression of T lymphocyte function was evident in individual animals at all stages of renal infection. The PHA responsiveness of lymphocytes from peripheral lymphoid tissue of pyelonephritic and control animals was similar, although some individual animals with renal infection did show increased responsiveness. Splenic T lymphocyte responses were low, compared with lymphocytes from peripheral lymphoid sites and the perirenal nodes and this relationship was maintained over the 28 days of the
FIGURE 69.

T cell function in the lymphoid tissues of animals with experimental pyelonephritis (E) and normal control animals (C). The PHA response of lymphocytes from various sites determined 3, 7, 14 and 28 days after infection and the ratio of the PHA responses expressed as a percentage. The stippled area represents the biological variation (coefficient of variation) in the PHA response of control animals.
days after renal infection
study. Lymphocytes from the perirenal node showed a greatly reduced PHA responsiveness compared with lymphocytes distant from the infection.

Active renal infection persisted for extended periods, but the changes in PHA responsiveness of lymphocytes were less marked during resolution of the lesion up to 28 days after challenge.

**PHA responsiveness of peripheral blood lymphocytes**

The response in culture to PHA stimulation of lymphocytes from individual pyelonephritic animals was assessed and compared with the response of peripheral blood lymphocytes obtained from normal animals analysed at the same time as the experimental group. Five groups of animals with five to nine animals in each group were sacrificed at selected intervals.

Apart from a decrease in the PHA responsiveness of lymphocytes from pyelonephritic animals 14 days after infection, there were no significant differences in the PHA responsiveness of lymphocytes from pyelonephritic animals and their matched controls (Figure 70).

**Functional capacity of lymphocytes in the cellular infiltrate of the infected kidney**

Four groups each of five animals were sacrificed at intervals over a 28-day period, and their kidneys removed. Regions containing pyelonephritic tissue were dissected away from the normal tissue. Suspensions of lymphocytes were prepared from these sections of tissue and their response to PHA examined in culture.

Although previous experiments indicated that the majority of the lymphocytes in the lymphocyte infiltrate were T cells, no
FIGURE 70.

T cell function in peripheral blood lymphocytes from animals with pyelonephritis compared with normal control animals. The results are expressed as the amount of tritiated thymidine incorporated by lymphocytes in culture following PHA stimulation.
cultures of lymphocytes obtained from pyelonephritic or normal kidneys over a 28-day period showed any response to stimulation with PHA. Cultures of lymphocytes obtained from other lymphoid sites in the pyelonephritic animals, however, responded normally to PHA stimulation.

**Influence of local factors in the kidney on the function of T cells**

In view of these unexpected results, experiments were initiated to explain the failure of lymphocytes from the cellular infiltrate of the kidney to respond to PHA stimulation. Suspensions of splenic lymphocytes were prepared from a normal animal and cultures established in the usual manner. Suspensions of kidney cells and cells from the lung of the same animal were then prepared in tissue culture medium and aliquots containing varying numbers of kidney cells added to the splenocytes in the insert medium. All cultures were then incubated and processed normally to determine the effect of the presence of kidney and lung cells on the response of splenocytes to PHA.

Addition of kidney cells to a culture of splenocytes from the same animal completely ablated the capacity of the splenocytes to respond to PHA. In four control cultures each containing $5 \times 10^6$ splenocytes, an average amount of $^3$H thymidine was incorporated to give 60,075 D.P.M./culture. In contrast, when $5 \times 10^6$ renal cells were added to the cultures, the average amount of $^3$H incorporated was only 90 D.P.M./culture. The PHA responsiveness of splenocytes in culture was restored as fewer renal cells were added to the culture, but even when the numbers of renal cells present were as low as 1%, significant depression of T cell function occurred.
DISCUSSION

These studies have shown that renal infection may alter the distribution of B cells in the peripheral blood and lymphoid tissue and affect the functional capacity of T lymphocytes in the spleen, particularly during the early stages of infection. The origin of lymphocytes forming the lymphocytic infiltrate in the kidney in pyelonephritis has been identified and an adverse effect of renal cells on the functional capacity of T lymphocytes in the kidney has been disclosed.

With methods available for identifying T and B cells, it is now possible to study changes in the population of lymphoid cells during the course of infection. This is important, as specific immune mechanisms are linked with either the T or B cell population of lymphocytes. Since the traffic of recirculating lymphocytes is a major factor in the induction of the immune response, any alteration to the pattern of lymphocyte recirculation may have a considerable effect on the nature of the host's response to infection.

The present studies have shown several consistent changes in the distribution of lymphocytes and, in particular, splenic lymphocytes, during the course of renal infection. The lymphocyte population in perirenal nodes showed an increase in B cells early in infection but no discernible changes were seen in the proportion of B cells in lymph nodes away from the infected kidney.

These studies too have demonstrated an increase in the percentage of B lymphocytes in the peripheral blood during the first seven days of experimental pyelonephritis and confirmed the
clinical studies showing that the B lymphocyte population is commonly elevated in the acute stages of bacterial infection (Niklasson & Williams, 1974). A number of other investigators have determined the effect of some of the more common infectious diseases on the distribution of T and B cells in man, although these studies have of necessity been confined to peripheral blood lymphocytes. The results of these reports have varied (Aiuti et al., 1973; Thorley, Smith & Sanford, 1974), but in general the more severe infections have resulted in an increase in the proportion of circulating B cells.

The present study has presumptively identified the majority of the lymphocytes in the lymphocytic infiltrate of the pyelonephritic kidney as T lymphocytes lacking surface immunoglobulin. Some of the lymphocytes classified as T cells on this basis may in fact be null cells (Williams et al., 1973) and Nossal's observation that antibody forming cells lose their surface immunoglobulins (Nossal & Lewis, 1972) will also need to be considered in view of the heterogeneity of the morphology of these lymphocytes (Cunningham, 1968). Absolute identification of the T lymphocytes will require a specific anti T cell serum and we are currently working on this problem.

The function of T cells in the inflammatory lesion of the pyelonephritic kidney is not known, but related studies suggest that T cells may be an important component of the inflammatory infiltrate. They have certainly been identified as the immunologically critical cells in Listeriosis and ectromelia virus infection where elimination of T lymphocytes removed effector activity from immune cell populations. Elimination of B lymphocytes or macrophages had no effect on
immunity to infection and showed clearly that the effector lymphocytes were T cells (Blanden, 1971; Lane & Unanue, 1972; Blanden & Langman, 1972; North, 1973; North, 1973a).

The use of surface markers to identify lymphocyte populations in lymphoid tissue does not reveal the functional capacity of these cells. An analysis of the activity of B cells in the kidney and lymphoid tissue during pyelonephritis has already been made (Miller, Smith & Sanford, 1971), and one of the objects of the present experiments was to determine the effect of renal infection on the ability of T lymphocytes to respond to stimulation. Three days after infection, a marked decrease in the functional capacity of splenic lymphocytes was found and the PHA responsiveness of splenocytes was reduced to 14% of normal. Lymphocytes from the perirenal nodes also showed reduced responsiveness whereas T cell function at lymphoid sites removed from the site of infection were similar in control and pyelonephritic animals.

The decrease in the functional capacity of T cells suggests either non-specific mobilization of T cells to the site of the lesion or a loss of function as a result of infection. Woodruff, investigating the effect of virus on T cell function, has found that the size of the circulating pool of thymus derived lymphocytes decreased rapidly in animals inoculated with Newcastle disease virus and suggested that the lymphocyte redistribution was due to the exposure of sialyl residues on the surface of the recirculating lymphocytes (Woodruff & Woodruff, 1974). Human lymphocytes treated with rubella virus in vitro also show a hypo-responsiveness to PHA (Olson et al., 1968), but the
effect of bacterial infection on T cell function in lymphoid tissue does not appear to have been studied. Although recruitment of T lymphocytes to the site of infection may be an explanation for the depressed responsiveness of splenic lymphocytes, it would seem that such a substantial reduction in functional capacity is more likely to be due to the suppressive effect of a factor, possibly analogous to cholera toxin which has been shown to suppress some functions of T cells (Warren et al., 1974).

The ability of renal cells to ablate the functional capacity of the thymus derived lymphocytes was an unexpected finding. Functional testing of lymphocytes is usually carried out on purified population of lymphocytes and in general the cellular environment from which they were obtained is not considered. The inference from these experiments is quite clear and suggests that although large numbers of T cells are present in the pyelonephritic kidney, they may be unable to elaborate soluble mediators or respond to contact with bacterial antigen. As a result, a potentially effective cell-mediated immune response would then be ablated by cells forming the cellular milieu. This may help to explain the persistence of infection in pyelonephritis, which remains one of the characteristic features of the disease, despite a seemingly adequate local immune response within the infected kidney. The phenomenon has been investigated further in the following chapter.
CHAPTER 12

NATURE OF THE FACTOR ASSOCIATED WITH

RENAL CELLS CAUSING ABLATION OF T LYMPHOCYTE FUNCTION
INTRODUCTION

In the previous chapter it was shown that the PHA responsiveness of normal lymphocytes in vitro could be blocked by the addition of normal kidney cells to the culture. The failure of T lymphocytes in the pyelonephritic kidney to respond to PHA was attributed to this phenomenon. The following experiments were carried out to characterize the factor causing ablation of the PHA response.

MATERIALS AND METHODS

The conditions were the same as those used in the previous chapter. In some experiments, a modification of the culture vessel was made to allow the lymphocytes in culture to be separated from the kidney cells or from the soluble extract of these cells. These experiments were carried out by incubating the lymphocytes on the membrane of an enlarged insert tube while the kidney cells were cultured on the membrane of a further insert tube suspended 3 mm. above the lymphocyte layer (Figure 71). Reservoir medium, added as usual, covered both insert tubes. A soluble fraction from kidney cells was prepared by disrupting a suspension of a known number of kidney cells using a MSE sonicator and an exposure time of two minutes. Complete rupture of the cells was confirmed by a microscopic examination of the suspension after sonication. The insoluble cell fraction was removed by centrifugation of the disrupted cell suspension at 3,000 r.p.m. for 15 minutes. The supernatant fluid was then sterilized by passage through a 0.45 µm cellulose acetate filter and stored at 4°C.
Single and double chamber culture vessels. In the double chamber vessel lymphocytes were cultured on the membrane of the larger insert tube while the kidney cells were cultured on the membrane of a further insert tube suspended 3 mm. above the lymphocyte layer. Culture medium was added to the reservoir so that the membranes of both tubes were covered.
Cell viability

A stock solution of 5 mg./ml. of fluorescein diacetate was prepared in acetone and stored in the deep freeze. The working solution was a 1:100 dilution of the stock solution in Hank's medium, prepared immediately prior to use and stored in the dark. The lymphocyte suspension was washed twice in Hank's medium and resuspended in 1 ml. of the medium. 0.1 ml. of the fluorescein diacetate working solution was added and the preparation kept in the dark at room temperature for 20 minutes. Gentle mixing by tapping was carried out at five minute intervals. The cell suspension was then washed twice in Hank's medium containing 20% foetal calf serum and finally resuspended in this medium. The number of fluorescing lymphocytes as a percentage of the total number of lymphocytes was then determined using a Zeiss microscope, stand 14, fitted with an ultraviolet light source and optics for fluorescence microscopy. Viable cells were seen as brightly fluorescing cells while the non-viable cells were only visible under white light optical conditions but were not fluorescent.

RESULTS

Ablation of the PHA response of rat lymphocytes by renal cells

Cultures containing $5 \times 10^6$ PHA stimulated splenic lymphocytes were prepared in triplicate and increasing numbers of a suspension of kidney cells from $0.009 \times 10^6$ to $5 \times 10^6$ were added to the culture. Cultures containing similar numbers of a suspension of lung cells were prepared in parallel. The effect of the addition of both the cell suspensions on the PHA response of splenic lymphocytes determined by $H^3$ thymidine uptake after three days in culture is shown in Figure 72. The experiments have shown that renal cells added to the PHA stimulated splenic lymphocyte
FIGURE 72.

Effect of varying numbers of kidney and lung cells on the PHA response of splenic lymphocytes. Tritiated thymidine incorporation by $5 \times 10^6$ lymphocytes, representing a 100% response in these experiments, was 58,000 c.p.m./$10^6$ lymphocytes. Each point is the mean of four individual experiments.
cultures depressed the PHA response when as few as 2% of the cells in culture were renal cells and total ablation of the PHA response was found with the addition of increasing numbers of kidney cells. In contrast, smaller numbers of the suspension of lung cells stimulated the PHA response of splenic lymphocytes and with higher concentrations of lung cells, 65% of the normal PHA response of splenic lymphocytes was found.

The ablative effect on kidney cells on the PHA response was not confined to rat splenocytes and when rat peripheral blood lymphocytes obtained from cervical and mesenteric lymph nodes were tested under the same experimental circumstances as the splenic lymphocytes, similar results were found (see Table 10).

**Cell-cell contact and kidney cell viability as a requirement for ablation**

Experiments were carried out to determine whether direct contact between the splenic lymphocytes and kidney cells was necessary to block the response of the lymphocytes to PHA. $5 \times 10^6$ splenic lymphocytes were cultured on the membrane of a large insert tube and increasing numbers of kidney cells from $0.62 \times 10^6$ to $5 \times 10^6$ were added to the second insert to be suspended 3 mm. above the lymphocyte culture. A marked reduction in the PHA responsiveness was found when renal cells were physically separated from the lymphocytes in culture using the twin insert chamber culture system. The PHA response of splenic lymphocytes under these conditions was reduced to 9% of normal (Figure 73). The necessity for viable kidney cells in the ablation of the PHA response of splenic lymphocytes was investigated in a standard culture vessel by preparing cultures in triplicate.
TABLE 10. Effect of ablation factor from $5 \times 10^6$ renal epithelial cells on the PHA response of rat lymphocytes obtained from the spleen, lymph nodes and peripheral blood.
<table>
<thead>
<tr>
<th>Lymphocyte source</th>
<th>DPM/10^6 lymphocytes</th>
<th>% reduction of PHA response</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10^6 splenocytes</td>
<td>83,204</td>
<td>0</td>
</tr>
<tr>
<td>5 x 10^6 splenocytes and ablation factor</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5 x 10^6 peripheral blood lymphocytes</td>
<td>59,482</td>
<td>0</td>
</tr>
<tr>
<td>5 x 10^6 peripheral blood lymphocytes and ablation factor</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5 x 10^6 lymphocytes (lymph nodes)</td>
<td>119,000</td>
<td>0</td>
</tr>
<tr>
<td>5 x 10^6 lymphocytes and ablation factor</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>
containing $5 \times 10^6$ splenic lymphocytes and adding to each series of cultures increasing numbers of viable and dead kidney cells and dead splenocytes. The cells were killed by immersion at $56^\circ C$ for 15 minutes. Total ablation of the PHA response was found with higher concentrations of both viable and dead kidney cells and similar effects were also found when decreasing numbers of cells were added (Figure 73). Killed splenocytes, assayed as a control for the effect of killed cells, led to a minor decrease in PHA responsiveness of splenic lymphocytes but only at the higher concentrations of dead cells. In an extension of this experiment, duplicate suspensions of varying numbers of renal cells were made. One sample from each duplicate was added directly to a culture of $5 \times 10^6$ PHA stimulated splenocytes and the other disrupted by sonication and the soluble fraction added to a duplicate culture of splenocytes. The sonicate showed a similar PHA ablative capacity as the intact renal cells (Figure 74).

**Cortical and medullary renal tissue**

Several features of renal infection have been ascribed to the characteristics of the cortical and medullary tissue. Experiments were carried out to assess the PHA ablative capability of renal cortex and medulla. Tissue dissection was carried out as described in Chapter 2. Renal cells from both cortex and medulla displayed equal ablative capacities when suspensions were incubated in culture with $5 \times 10^6$ splenic lymphocytes (Table 11).

**Is the ablation factor a non-specific cell toxin?**

In a previous experiment, some ablation was found when renal
FIGURE 73.

Effect of varying numbers of viable kidney cells, killed kidney cells, killed splenocytes and viable kidney cells separated from the lymphocytes by a dialysis membrane on the PHA response of splenic lymphocytes.
The graph illustrates the percent of splenic lymphocyte response (78,000 D.P.M./10^6 lymphocytes) as a function of the number of cells added to cultures of 5x10^6 splenocytes. The data is represented by different symbols:

- X-X: killed renal cells
- Δ: viable renal cells
- ●○○: killed splenocytes
- ○○○: viable renal cells

The graph shows that the percent of response increases as the number of cells added increases. The curves for killed renal cells and viable renal cells diverge, indicating a significant difference in the response.
FIGURE 74.

Comparative effect of viable kidney cells and the soluble supernatant from disrupted renal cells on the PHA response of splenic lymphocytes.
<table>
<thead>
<tr>
<th></th>
<th>DPM/10^6</th>
<th>% of normal response</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10^6 splenic lymphocytes</td>
<td>102,745</td>
<td>100</td>
</tr>
<tr>
<td>5 x 10^6 splenic lymphocytes plus 5 x 10^6 unselected renal cells</td>
<td>602</td>
<td>0.9</td>
</tr>
<tr>
<td>5 x 10^6 splenic lymphocytes plus 5 x 10^6 renal cortical cells</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 x 10^6 splenic lymphocytes plus 5 x 10^6 renal medullary cells</td>
<td>1516</td>
<td>1.1</td>
</tr>
</tbody>
</table>
cells were separated from PHA stimulated lymphocytes in a twin membrane culture system. This suggested that a small molecular weight toxic material or possibly the accumulation of a high concentration of potassium and other ions might be responsible for the loss of PHA responsiveness. Further experiments were carried out to investigate this.

(a) Dialysis: Supernatant material from the sonication of $5 \times 10^6$ renal cells was dialysed for 24 hours in 0.01M phosphate buffer. The ablative capacity of this material was then tested in culture with $5 \times 10^6$ splenic lymphocytes in parallel with the non-dialysed material. The ablative capacity of the sonicate was not reduced by dialysis (Table 12). The experiment has shown that in addition to ablative material capable of passing through the dialysis membrane in the double chamber experiment, ablative material of a larger molecular weight is also present. This result was confirmed in a further experiment where the dialysed material was added to the inner insert of a double chamber system. Addition of ablation factor to the interior chamber did not affect the PHA response of the lymphocytes.

(b) The PHA response as an index of viability: If the effect of the ablative material was mediated through an effect of the material on lymphocyte viability, it was reasoned that addition of the ablative factor at intervals after the initiation of the culture would affect cell viability and prevent the incorporation of tritiated thymidine when it was added to the lymphocytes after 72 hours of culture.

Ablation factor from $5 \times 10^6$ kidney cells was added to cultures of PHA stimulated splenocytes immediately and 24, 48 and 72
TABLE 12. The effect of dialysis and the use of a double membrane culture vessel on the activity of ablation factor.
<table>
<thead>
<tr>
<th>Standard Culture Vessel</th>
<th>DPM/10^6 lymphocytes</th>
<th>% of normal response</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10^6 splenocytes</td>
<td>69,088</td>
<td>100</td>
</tr>
<tr>
<td>5 x 10^6 splenocytes</td>
<td>29</td>
<td>0.04</td>
</tr>
<tr>
<td>plus ablation factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 x 10^6 splenocytes</td>
<td>42</td>
<td>0.06</td>
</tr>
<tr>
<td>plus ablation factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(dialysed 24 hours)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Double Membrane Vessel</th>
<th>DPM/10^6 lymphocytes</th>
<th>% of normal response</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10^6 splenocytes</td>
<td>31,370</td>
<td>100</td>
</tr>
<tr>
<td>in a double membrane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>culture vessel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 x 10^6 splenocytes</td>
<td>29,080</td>
<td>93</td>
</tr>
<tr>
<td>plus ablation factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>in the internal chamber</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
hours after establishing the culture. A decreasing effect was found as the interval between the commencement of culture and the addition of ablation factor increased (Figure 75). The results have shown that complete ablation of the PHA response could only be achieved if the factor was added at the initiation of the culture. PHA stimulated lymphocytes in culture which had been in contact with ablation factor for up to 48 hours still incorporated tritiated thymidine at the end of the culture period.

(c) Specificity for rat lymphocytes: Further evidence against a non-specific toxic effect was provided in an experiment where ablation factor was added to cultures of human and rat peripheral blood lymphocytes. A 100-fold difference in the ability of ablative factor to block the PHA responsiveness of rat peripheral blood lymphocytes was noted when this function was compared with the effect on human peripheral blood lymphocytes under the same experimental conditions (Table 13).

(d) Cell viability: Cultures containing $5 \times 10^6$ lymphocytes and ablation factor from $5 \times 10^6$ renal cells were incubated for up to 24 hours under the normal cultural conditions. Neither cell numbers nor cell viability determined by fluorescein diacetate uptake was affected and when lymphocytes in contact with ablation factor were compared with a control culture under the same conditions, no differences were found.

(e) Heat stability of ablation factor: Heat stability tests were carried out to gain some insight into the physical and chemical characteristics of the ablating factor. Supernatant material from $5 \times 10^6$ renal cells was heated at temperatures
FIGURE 75.

PHA response as an index of cell viability in the presence of ablation factor. Ablation factor from 5 x 10^6 kidney cells was added to cultures of PHA stimulated splenocytes immediately, and 24, 48 and 72 hours after establishing the culture.
hours in culture of P.H.A. stimulated lymphocytes before the addition of ablation factor
TABLE 13. Addition of ablation factor from $1 \times 10^6$ kidney cells to an equivalent number of PHA stimulated rat and human peripheral blood lymphocytes.
<table>
<thead>
<tr>
<th></th>
<th>Human P.B.L.</th>
<th>Rat P.B.L.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C.P.M./10⁶</td>
<td>% of normal response</td>
</tr>
<tr>
<td></td>
<td>lymphocytes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>44,000</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>68,490</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>58,300</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>40,200</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>1 x 10⁶ PBL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5 x 10⁶ PBL plus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cells</td>
<td></td>
</tr>
<tr>
<td>TABLE 14. Heat stability of ablation factor. Ablation factor from 5 x 10^6 splenic lymphocytes was held for 15 minutes at temperatures from 45 to 110°C. before being assayed in cultures containing 5 x 10^6 splenic lymphocytes.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment of ablation factor</td>
<td>DPM/10^6 lymphocytes</td>
<td>% of normal response</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>----------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>5 x 10^6 splenocytes</td>
<td>44,833</td>
<td>100</td>
</tr>
<tr>
<td>5 x 10^6 splenocytes plus ablation factor from 5 x 10^6 renal cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>unheated</td>
<td>423</td>
<td>0.9</td>
</tr>
<tr>
<td>heated at 40°C for 15 minutes</td>
<td>256</td>
<td>0.6</td>
</tr>
<tr>
<td>heated at 56°C for 15 minutes</td>
<td>265</td>
<td>0.6</td>
</tr>
<tr>
<td>heated at 80°C for 15 minutes</td>
<td>11,781</td>
<td>26</td>
</tr>
<tr>
<td>heated at 100°C for 15 minutes</td>
<td>11,461</td>
<td>25</td>
</tr>
<tr>
<td>heated at 110°C for 15 minutes</td>
<td>9,621</td>
<td>21</td>
</tr>
</tbody>
</table>
from 45 to 110°C for 15 minutes, and after centrifugation to deposit the flocular precipitate, the ablative capacity was tested in cultures containing $5 \times 10^6$ splenic lymphocytes. The results have shown that the ablative factor was remarkably resistant to heat. Even after exposure to 110°C in the autoclave for 15 minutes, 80% of the ablative capacity was retained (Table 14).

**DISCUSSION**

The experiments have demonstrated that the response of rat lymphocytes to PHA can be ablated entirely by normal kidney cells. Even when the number of kidney cells added to cultures of splenic lymphocytes were less than 2% of the cells present, some ablation of T cell function could be detected. Viable kidney cells were not essential and it has been possible to demonstrate ablation of the PHA response by a soluble product from disrupted kidney cells which has been called "ablative factor". The experiments adequately explain the puzzling result from the previous experiments where T lymphocytes were found to be the predominant lymphocyte in the inflammatory infiltrate yet it was not possible to demonstrate any response of the lymphocytes to phytohaemagglutinin.

This phenomenon may help to explain the persistence of infection in pyelonephritis which is one of the characteristic features of the disease despite a seemingly adequate local immune response within the infected kidney (Lehmann et al., 1969; Miller & North, 1971; Miller, Smith & Sanford, 1971; Spencer & Fairhead, 1972).
The relevance of these results to clinical and experimental transplant immunology are also obvious and the renal environment may well be found to influence the immune capabilities of invasive lymphocytes. An indication that this may be the case can be found in a report by Ivanyi et al. (1973) where T lymphocytes isolated from a local inflammatory lesion were examined in culture using PHA responsiveness as an index of function. No PHA induced mitogenesis of the T lymphocytes could be demonstrated.

The chemical nature of ablation factor has not been determined but on the basis of reported studies of related factors isolated from tissues, it appears to be unique. Several workers have described T lymphocyte chalones which have been isolated from pig lymph nodes (Moorhead et al., 1969), guinea pig spleen (Ernstrom & Nordlind, 1974), rat lymph nodes and spleen (Houck, Irausquin & Leikin, 1971). These compounds have been characterized as macro-molecules of 30,000 to 75,000 Daltons (Houck et al., 1971; Jones, Paraskova-Tchernozemska & Moorhead, 1970; La Salvia, Garcia-Giralt & Macieira-Coelho, 1970) but in contrast to the present factor, they have been found to be thermolabile and not stable on long-term storage. Furthermore, chalones by definition are tissue specific but not species specific inhibitors present in, and produced by tissue, the proliferation of which they specifically inhibit (Bullough & Rytomaa, 1965; Mathe, 1972).

A related substance affecting the function of lymphocytes has been isolated from the liver by Schumacher et al. (1974) but this inhibitor also does not resemble the factor described in
these experiments. Schumacher et al. have identified their inhibitor as a protein but the remarkable heat stability of ablation factor makes it unlikely that it will be found to be a protein. Furthermore, no species specificity could be found for the inhibitor from liver whereas ablation factor has been shown to be specific for rat lymphocytes and did not affect the PHA response of human peripheral blood lymphocytes.

Although many characteristics of the ablation factor remain to be defined, there is sufficient evidence to differentiate it from related substances and this suggests that it may be important in modifying the function of lymphocytes in the kidney. Indeed, already confirmatory evidence has been presented in a study of the function of T lymphocytes in the kidneys of rabbits. Smith et al. (1974, personal communication), using uropod formation (Rosentreich et al., 1972) were able to identify T lymphocytes as a significant proportion of the lymphocytes in infected kidneys but also found that the T lymphocytes were totally unresponsive to PHA.

The possibility that ablation factor is a non-specific lymphotoxic agent has been excluded and in preliminary tests high concentrations of ablation factor were not cytolytic to lymphocytes after an exposure period of 24 hours. There is also evidence for specificity and although ablation factor has a marked effect on the PHA responsiveness of rat lymphocytes, human lymphocytes were not affected to the same degree. B lymphocyte function in the rat kidney is also not affected and immunoglobulin synthesis within the infected kidney has been adequately documented (Lehmann et al., 1968; Miller & North,
Lymphocytes held in contact with ablation factor for up to 24 hours showed normal viability and the addition of the factor to PHA stimulated lymphocyte cultures at varying intervals after the initiation of the culture did not ablate the PHA response. This strongly suggests that the ablation factor is not simply a lymphotoxin as cells in contact with the factor for up to 48 hours were still able to incorporate tritiated thymidine when this nucleotide was added to the culture. An alternative interpretation of this result is that the PHA stimulated lymphocytes were resistant to lymphotoxic substances once blastogenesis was induced.

Although further studies are required to chemically characterize the factor, these experiments have provided basic information on the biological characteristics of ablation factor and extend the original observations which have explained the failure of T lymphocytes in the kidney to respond to PHA.
CHAPTER 13

SELECTIVE DEFICIENCY OF THYMUS DERIVED LYMPHOCYTES IN PYELONEPHRITIS
INTRODUCTION

Studies of the host defence system in renal infection have included investigations of non-specific immune mechanisms (Jackson, Arana & Kozij, 1965), local factors affecting the immune response (Chernew & Braude, 1962; Beeson & Rowley, 1959) and the protective role of humoral immunity in renal infection (Radford et al., 1974; Kaijser & Olling, 1973; Brooks, Lyons & Braude, 1974).

In Chapters 11 and 12 it was observed that renal cells added to normal PHA responsive rat lymphocytes could ablate the ability of the T lymphocytes to respond to stimulation by PHA. The results suggested that cell-mediated immunity may be an important host immune mechanism in pyelonephritis and that the inability of T lymphocytes to function in the renal environment may be a factor contributing to the persistence of infection.

In the following experiments, the role of T lymphocytes in the immunobiology of pyelonephritis has been explored. Renal infection was induced in experimental animals deprived of T lymphocytes and the effect of T cell ablation on the bacteriological, pathological and immunological features of the disease determined.

MATERIALS AND METHODS

Animal strain

Female animals were obtained from a random bred strain of Wistar rat.
Production of infection
Pyelonephritis was produced by the direct inoculation of E. coli 075 into the exposed kidney using a glass microcapillary. A total of $3 \times 10^5$ organisms suspended in normal saline was injected into the kidney in each of three injection sites (Chapter 2).

Bacterial content of renal tissue
Nutrient agar pour plates of serial ten-fold dilutions of homogenized kidney were made to obtain the bacterial count per gram of wet renal tissue.

Haematological screening
Blood was collected from the tail into heparinized capillary tubes and diluted immediately in Isoton. Samples were processed in a Coulter counter, Model S, and the total leucocyte count, haemoglobin, red blood cell count, packed cell volume and red blood cell indices determined. A blood film for differential leucocyte count, and red blood cell morphology was also examined.

Collection of blood for antibody titres
See Chapter 8.

The preparation of T lymphocyte deprived animals, the in vitro assay of PHA stimulated mitogenesis of thymus derived lymphocytes and the determination of serum antibody levels have been described previously (Chapters 9 and 10).

Statistical analysis
Analysis of the histologic and bacteriologic data was carried out using the Wilcoxon sum of ranks analysis for non-parametric data.
Experimental design
These experiments were designed to assess the effect of T cell ablation on the course of acute, resolving and chronic E. coli renal infection. Pyelonephritis was induced in thymectomized and irradiated T cell deprived animals (TXIR), animals that had been irradiated only (IR), and a control unmanipulated group. The gross pathology, histopathology, bacteriology and the humoral immune response to infection was compared in animals from the three groups 3, 14 and 28 days after the induction of pyelonephritis.

RESULTS
Pyelonephritis
Injection of E. coli 075 into the kidney with a glass microcapillary resulted in a consistent and reproducible infection in the renal parenchyma. Full details have been given in Chapter 2.

Haematological changes in the experimental model
Baseline haematological values were determined in four groups each of six experimental animals (TXIR, IR, Sham TXIR and normals) and subsequently at regular intervals following thymectomy and irradiation. Haemoglobin and red blood cell numbers remained at normal levels in all groups over the period of the experiments. Total leucocyte numbers, however, were reduced from a mean of 11,000 to 6,000/cu. mm. when the TXIR and IR groups were compared with the sham thymectomized and control groups. This was an absolute lymphopenia as a result of irradiation and a similar reduction in lymphocyte numbers was found in both the TXIR and IR groups.
T cell response to PHA stimulation in thymectomized and irradiated animals (TXIR)

Fourteen days after the final irradiation the mitogenic response to PHA of splenic lymphocytes from the TXIR, IR and control rats was determined. At the time the rats were killed the thorax of each thymectomized animal was examined for thymic remnants.

The PHA response of T cells in cultures of splenocytes from the thymectomized and irradiated animals was almost entirely ablated. The (3H) thymidine incorporation by PHA stimulated spleen cells from thymectomized and irradiated (TXIR), irradiated (IR) and normal rats are shown in Figure 76. A mean 43,000 DPM/10^6 lymphocytes was found when the amount of (3H) thymidine incorporation was determined for six PHA stimulated normal splenocyte cultures. In contrast, the comparable mean figure for the culture of splenocyte suspensions from six TXIR animals was 444 DPM. The results from sham thymectomized animals were similar to the normal animals, but a reduction in PHA responsiveness of splenocytes from irradiated animals was found. All animals remained in excellent health throughout the experiment and showed normal activity and weight gain.

Sublethal irradiation and the humoral immune response

The objective in these experiments was to produce an experimental animal with an ablated T cell response but capable of developing a normal humoral immune response. The effect of irradiation alone on the ability of the treated animals to produce circulating antibacterial antibody was determined by irradiating animals with 250 rads at 2-weekly intervals until
FIGURE 76.

The comparative PHA responsiveness of splenic lymphocytes from T lymphocyte deprived (TX-IR) irradiated only (IR) and sham thymectomized (SH-TX) animals. The results are the mean and standard error from 6 animals in each group compared with a mean response of 43,000 DPM per $10^6$ splenic lymphocytes from unmanipulated animals analysed at the same time.
a total of 1,000 rads had been given. Pyelonephritis was induced in five groups of ten animals which were challenged at intervals from 24 hours to 14 days after the final irradiation. The humoral immune response (Figure 77) shows that circulating antibody production was depressed in those groups challenged up to seven days after irradiation but returned to normal in the group challenged 14 days after the final irradiating dose. In subsequent experiments where the role of T cells in infection was assessed, an interval of 14 days was allowed between the final irradiation and the induction of pyelonephritis.

**Gross pathology**

The effect of T cell ablation on the gross pathological changes during the course of pyelonephritis are shown in Figure 78. Characteristic lesions were found in all the pyelonephritic kidneys into which bacteria had been introduced. There were no significant differences between the three groups when the extent of the acute infection was assessed three days after challenge.

Fourteen days after challenge, resolution of the pyelonephritic lesions had commenced and a similar degree of scar formation was found in all groups. When the degree of residual infection was compared, animals in the TXIR group were found to have significantly fewer ($P = 0.01$) unresolved lesions than the control groups. Twenty-eight days later, extensive scar formation had occurred in the infected kidney of pyelonephritic animals from all groups. There were no significant differences in the extent of scar formation between the different groups.
FIGURE 77.

Humoral immune response in pyelonephritic animals when infection was induced at increasing intervals up to 14 days after the final irradiation.
FIGURE 78.

Gross pathology in T lymphocyte deprived (TXIR), irradiated (IR) and control animals 3, 14 and 28 days after the induction of pyelonephritis. The term residual infection, Res Inf, was used to indicate foci of incompletely healed lesions within scarred areas (see Chapter 2).
and although the animals in the TXIR group appeared to have fewer unresolved lesions than the control animals, the differences were not statistically significant. Criteria for quantitating the degree of scar formation have been given in Chapter 2.

**Bacteriology**

Bacterial numbers in the kidney of pyelonephritic animals were quantitated 3, 14 and 28 days after the induction of unilateral infection in TXIR, IR and normal animals (Figure 79). Three days after challenge the bacterial content of acutely infected kidneys in all groups were similar. Fourteen days later, when the lesions had begun to resolve, bacterial numbers had decreased in all animals including the TXIR group. Bacterial numbers in the kidney of animals in the TXIR group were in fact significantly lower than either the control group or the irradiated group ($P = 0.02$). Twenty-eight days after infection, there were no significant differences in the number of bacteria in the chronically infected kidney from the three groups.

**Histopathology**

Histopathological changes occurring in the kidney during the course of renal infection in T cell ablated animals were compared to the histopathology of pyelonephritis in normal animals. Serial histological sections were examined to determine quantitatively the extent of renal damage (see Chapter 2), the intensity of lymphocytic infiltration in the pyelonephritic lesion and to assess the proportion of lymphocytes in the infiltrating cells. The effect of T cell ablation
FIGURE 79.

Bacterial numbers in the kidney of T lymphocyte deprived (TXIR), irradiated (IR) and control animals 3, 14 and 28 days after the induction of renal infection with E.coli.
on the inflammatory polymorphonuclear infiltrate and the degree of fibrosis in the scarred areas were also examined.

Three days after challenge, the degree of renal damage and the cellular infiltrate, assessed by the examination of serial sections of each infected kidney were similar in all groups (Table 15). The cellular infiltrate in the acute lesion of all three groups consisted mainly of polymorphonuclear leukocytes but mononuclear cells were also present.

In the resolving lesion 14 days after infection the polymorphonuclear infiltrate had been replaced by an intense mononuclear infiltrate. Histopathologically, the degree of renal damage was again similar in all groups and there was no significant difference in the degree of round-cell infiltration between the control and TXIR groups. Twenty-eight days after challenge, resolution of the lesions was comparable in all groups and T cell ablation had not led to any significant extension of the lesions. A variation in the intensity of the lymphocytic infiltrate was found when the pyelonephritic lesions in the kidney of the TXIR group were examined and compared with their controls. In individual animals from the TXIR group, there was a marked paucity of lymphocytes in the lymphocytic infiltrate while in others the intensity of the lymphocytic infiltrate was comparable to the controls. In the irradiated animals, the mononuclear infiltrate was also comparable to the controls but fewer of the infiltrating cells were lymphocytes.

Serum antibody levels in pyelonephritis

Renal infection was induced in three groups each of six
TABLE 15. Histopathological changes in the kidney of pyelonephritic animals 3, 14 and 28 days after renal infection.
<table>
<thead>
<tr>
<th></th>
<th>Degree renal damage*</th>
<th>Degree mononuclear infiltrn.</th>
<th>% infiltrate mono lympho cyte</th>
<th>Degree renal damage</th>
<th>Degree mononuclear infiltrn.</th>
<th>% infiltrate mono lympho cyte</th>
<th>Degree renal damage</th>
<th>Degree mononuclear infiltrn.</th>
<th>% infiltrate mono lympho cyte</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Mean</td>
<td>7.4</td>
<td>5.9</td>
<td>36</td>
<td>63</td>
<td>7.0</td>
<td>6.0</td>
<td>26</td>
<td>73</td>
<td>4.2</td>
</tr>
<tr>
<td>Std. error</td>
<td>0.46</td>
<td>0.53</td>
<td>5.9</td>
<td>5.9</td>
<td>0.38</td>
<td>0.40</td>
<td>4.1</td>
<td>4.1</td>
<td>0.75</td>
</tr>
<tr>
<td><strong>IR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Mean</td>
<td>6.5</td>
<td>4.2</td>
<td>39</td>
<td>61</td>
<td>7.1</td>
<td>5.1</td>
<td>42</td>
<td>58</td>
<td>5.4</td>
</tr>
<tr>
<td>Std. error</td>
<td>0.63</td>
<td>0.38</td>
<td>4.8</td>
<td>4.8</td>
<td>0.48</td>
<td>0.41</td>
<td>4.4</td>
<td>4.4</td>
<td>0.71</td>
</tr>
<tr>
<td>Signific.** NS</td>
<td>P.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>P.02</td>
<td>P.02</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>IR &lt; Con</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Con &lt; IR</td>
<td>Ir &lt; Con</td>
<td></td>
</tr>
<tr>
<td><strong>TXIR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Mean</td>
<td>6.14</td>
<td>4.71</td>
<td>44</td>
<td>55</td>
<td>6.5</td>
<td>4.3</td>
<td>32</td>
<td>67</td>
<td>7.1</td>
</tr>
<tr>
<td>Std. error</td>
<td>0.76</td>
<td>0.47</td>
<td>7.1</td>
<td>7.1</td>
<td>0.48</td>
<td>0.43</td>
<td>4.9</td>
<td>4.9</td>
<td>0.42</td>
</tr>
<tr>
<td>Signific.** NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Quantitative 0-10 scale used to make a comparative assessment of the cellular infiltrate and degree of renal damage. Refer to Chapter 2 for details.

** Degree of significance when compared to control animals (pyelonephritic non-manipulated animals) using the Wilcoxon sum of ranks analysis.
animals (TXIR, IR and controls) 14 days after the final irradiation. Serum samples were obtained 3, 7, 14 and 28 days after challenge and antibody levels determined. Neither T cell ablation nor irradiation alone had any effect on the specific immune response to infection (Figure 80).

Lymphocyte numbers at local sites
The effect of the loss of T cells on the number of lymphocytes at local and peripheral lymphoid sites during the course of infection was investigated in TXIR, IR and control animals, each with six rats per group. Animals were killed 3, 14 and 28 days after the induction of renal infection and the perirenal node, cervical node, lumbar node, and spleen placed in chilled tissue culture medium. Single cell suspensions were prepared and cell counts carried out in a Neubauer bright line haemocytometer. Despite the absence of PHA responsive T cells in the TXIR group and an absolute lymphopenia at the time of challenge, lymphocyte numbers at all the lymphoid sites studied were within 10% of the cell numbers found in lymphoid tissue at the same site of pyelonephritic but otherwise normal animals (Table 16).

When the number of lymphocytes in the enlarged renal lymph node from the pyelonephritic T cell ablated animals were compared with lymphocyte numbers in renal nodes from pyelonephritic but otherwise normal animals, little difference was found.

DISCUSSION
The cell-mediated immune response to infection depends on the functional activity of the thymic dependent T lymphocytes. As
FIGURE 80.

Serum antibody titres in T lymphocyte deprived (TXIR), irradiated (IR) and control animals determined by passive haemagglutination. These data are the mean titres of the six animals in each group sampled 3, 7, 14 and 28 days after the induction of pyelonephritis.
TABLE 16. Total lymphocyte numbers in representative lymphoid tissue from thymectomized and irradiated animals (TXIR) and irradiated animals (IR) compared with normal control animals 3, 14 and 28 days after the induction of renal infection.
<table>
<thead>
<tr>
<th>Lymphoid tissue</th>
<th>3 days TXIR</th>
<th>3 days IR</th>
<th>28 days TXIR</th>
<th>28 days IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perirenal nodes</td>
<td>94 98</td>
<td>90 95</td>
<td>102 101</td>
<td></td>
</tr>
<tr>
<td>Cervical nodes</td>
<td>95 97</td>
<td>106 106</td>
<td>97 97</td>
<td></td>
</tr>
<tr>
<td>Lumbar nodes</td>
<td>85 93</td>
<td>98 96</td>
<td>99 97</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>97 97</td>
<td>100 100</td>
<td>95 96</td>
<td></td>
</tr>
</tbody>
</table>
a result of specific immunologic stimulation, antigen committed T cells replicate and elaborate a number of soluble mediators which expand the number and type of cell involved in the inflammatory reaction (David, 1971). The activity of T lymphocytes can be monitored by a number of in vitro assays and there is a great deal of evidence to support the concept that the cellular responses in culture simulate those of functional T lymphocytes in the intact animal (Selected authors, 1972; Bloom & Glade, 1971). In these experiments, the effect of ablation of T cells by adult thymectomy and serial sublethal irradiation on the course of experimental renal infection was investigated. The PHA response was reduced to less than 1% of normal and confirmed the suitability of the experimental model for assessing the role of T lymphocytes in pyelonephritis. The marked reduction in the number of PHA responsive T lymphocytes did not alter the disease process and when the gross pathology, histopathologic and bacteriologic features of pyelonephritis in T cell ablated animals were compared with non-manipulated pyelonephritic animals, no major differences were found.

With the differentiation of specific immunologic effector mechanisms into T and B cell dependent compartments, the relative importance of humoral and cell-mediated immune responses in the immunobiology of infectious diseases is now being investigated. In some instances, the information has been obtained from clinical studies of patterns of infection in immunodeficient individuals but in most cases the details have come from manipulations of the immune responsiveness of experimental animals prior to the induction of infection.
In the present experiments, T cell deprivation and ablation of the cell-mediated immune response had a remarkably slight effect on the pathology of the pyelonephritic lesion. Neither the degree of macroscopic scarring and resolution of the lesions nor the histopathological examination suggested an adverse effect when lesions from T cell-deprived and intact pyelonephritic animals were compared. The number of organisms persisting in the pyelonephritic kidneys were also similar in the three groups studied, and after 14 days, there were even fewer organisms present in the kidneys of animals lacking a cell-mediated immune response.

T cell ablation likewise did not affect the ability of the host to produce circulating antibody following renal infection. Several authors have investigated the effect of thymectomy alone on the immune response to bacterial and other antigens and their common finding that thymectomy did not affect the humoral immune response (Barnett, Souda & Sanford, 1963; Aisenberg & Wilkes, 1964) is consistent with the extended life span of the T cell population. Even though care was taken in the present experiments to eliminate the residual T cell population remaining after thymectomy, the ability of the host to produce circulating antibody following renal infection was not impaired, and serum antibody levels in the T cell deprived animals were similar to those in the control groups. These results are consistent with the T cell independence of the immune response to lipopolysaccharides of Gram negative bacilli reported by other authors (Andersson & Blomgren, 1971; Manning, Reed & Julita, 1970). Although a regulatory effect
on the immune response to bacterial antigen by T cells has been proposed (Baker et al., 1970; Kerbel & Eidinger, 1972), the results have not been confirmed by other investigators (Barth, Singla & Ahlers, 1973; Veit & Michael, 1972) or supported by our own data.

Division of the immune response into cell-mediated and humoral components has led to attempts to manipulate the immune response of experimental animals to determine the effective mechanism associated with specific infectious disease. Several approaches have been used including the use of immuno-suppressive drugs and the restoration of immune competence by the transfer of selected cellular components to lethally irradiated animals. The results from some of these experiments have been tabulated in Table 17. Many of the experiments have given unequivocal results although most authors have been cautious in claiming that the immune mechanisms identified has been the sole significant factor in host protection. In some cases, conflicting evidence has been found, but a number of concepts have emerged that have defined patterns of resistance to infectious diseases in experimental animals and in man.

Infection with enterovirus, coxsackie, asian influenza, rabies and yellow virus appear to be controlled by humoral immune mechanisms whereas cell-mediated immunity is of primary importance in infections caused by pox, herpes, myxo and the tumor-inducing viruses where the viral antigens are expressed on the cell membrane or are formed by budding from the cell membrane. Components of the immune response to bacterial infections have also been investigated, but the results have
TABLE 17. Experimental determination of immune mechanisms in infectious diseases.
<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Effective immune mechanism</th>
<th>Evidence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avian influenza</td>
<td>Humoral immunity (B cells)</td>
<td>Bursectomy in chickens was associated with a significantly higher recurrence of temp, elevation and mortality. Thymectomy had no significant effect on virus infection.</td>
<td>Portnoy (1973) Cell Immunol. 9, 251.</td>
</tr>
<tr>
<td>Fowl-pox</td>
<td>Cell mediated more than humoral</td>
<td>Influence of thymectomy and bursectomy studied. CMI plays a more important role in recovery than humoral immunity but the latter cannot be disregarded.</td>
<td>Morita (1973) J.Immunol. 111, 1495.</td>
</tr>
<tr>
<td>Coxsackie</td>
<td>Humoral antibody</td>
<td>100% mortality following cyclophosphamide treatment, nil mortality cyclo &amp; antibody. Nil affected by thymectomy.</td>
<td>Rager-Zisman &amp; Allison (1973)</td>
</tr>
<tr>
<td></td>
<td>T cell initiated immunity</td>
<td>T cells and a radiosensitive recipient (Blood monocyte) component was necessary for the full expression of the antiviral activity of both immune cells and sera.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mediated through mononuclear effector cells.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>Mononuclear cell invasion of infected liver foci triggered by CMI was of key importance in recovery from primary mouse pox.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immune cell transfer promoted denser packing of mononuclear cells and regression of the infectious foci.</td>
<td></td>
</tr>
</tbody>
</table>
been less clear with the exception perhaps of the intracellular bacteria. As a result, there are few precedents that would allow one to predict the effect of T cell ablation on the course of renal infection. Persistence of infection for prolonged periods has been a feature of pyelonephritis and many host factors including the immune response to infection have been proposed as contributing to bacterial persistence and the pathology of chronic renal infection.

The role of cell-mediated immunity in renal infection has been investigated by at least two other groups. Zaruba found that prior sensitization of experimental animals did not favourably affect the course of infection in rabbits and infection was established more frequently in the presensitized group (Zaruba, Vejboras & Chobola, 1971). Bailey et al. on the other hand, have interpreted their results showing an increase in leucocyte migration in some patients with pyelonephritis as possibly indicating a defective cell-mediated immunity (Bailey et al., 1974).

The assay of PHA responsiveness used in these experiments to determine the extent of T cell depletion, however, is only an indicator of potential T lymphocyte function, and a reduction of PHA responsiveness, even as low as 1% of normal, can only be taken as presumptive evidence for the ablation of T cell function. Several lines of investigation have provided evidence for the existence of a heterogeneity among the peripheral T lymphocytes (Cantor & Asofsky, 1972; Cantor & Asofsky, 1970; Asofsky, Cantor & Tigelaar, 1971) and it is possible that an effector T lymphocyte population may have been left
intact after the experimental manipulations. This is considered unlikely, however, in view of the fact that adult thymectomy and irradiation are known to abolish the activity of the short-lived radiation sensitive T lymphocytes which have been shown to be involved in the inflammatory response to bacterial infection (Stobo, 1972; McGregor, Koster & MacKaness, 1971 and 1970).

With the recent demonstration of both T cell and B cells in the cellular infiltrate in pyelonephritis (Smith, Adkins & McCreary, 1974 and Chapter 11), the effect of each of the lymphoid cell populations on the function of the individual components will need to be considered. T cell modulation of B cell function by suppressor cells has been described (Katz & Benacerraf, 1972) and B cell modulation of the T cell response has been observed (Turk & Parker, 1973). So far, the relationship between the humoral and cell-mediated immune responses in the local defence mechanisms of the kidney has not been determined although it may clearly be of importance.
CHAPTER 14

B CELL ABLATION. IMMUNOLOGICAL ENHANCEMENT AS A FACTOR IN CHRONIC RENAL INFECTION
INTRODUCTION

One of the most consistent features of renal infection in man and the experimental animal is the demonstration of a specific immune response directed against the invading organism. There are many reports on the systemic immune response to infection, and recently, reports by the writer and others have described an immune response within the kidney that results in the local production of immunoglobulins with antibacterial activity (Lehmann et al., 1968; Miller & North, 1971; Miller, Smith & Sanford, 1971). The relationship of this immune response to the pathogenesis and immunobiology of pyelonephritis, however, remains controversial and obscure.

In the previous chapter, the role of T lymphocytes in host defence mechanisms was investigated. In the following experiments, the effect of the humoral immune (B lymphocyte) response on the course of renal infection has been studied by manipulating the ability of the host to respond to infection. Animals with renal infection were treated with selected immunosuppressive agents and the effect of treatment on the immunological, bacteriological and pathological features was determined.

Neither 6-mercaptopurine nor thiamphenicol significantly affected the outcome of renal infection, but a surprising result emerged when animals with pyelonephritis were treated with the immunosuppressive drug, cyclophosphamide. Although the animals were unable to mount a humoral immune response against the invading organism, many of the pyelonephritic lesions were sterile and significantly fewer bacteria
persisted in the kidney of cyclophosphamide treated animals compared with untreated pyelonephritic animals.

The interpretation of these results is that the immune response to renal infection, rather than favouring the host's capacity to eliminate the invading organism, plays an immuno-protective role protecting the bacterial cell from normally effective host defence mechanisms. The role of immuno-enhancing antibodies has been investigated extensively in tumour and transplantation immunology but it is believed that this is the first report involving enhancement as a factor in the persistence of bacterial infections.

MATERIALS AND METHODS

Animal strains

Female rats weighing 220 to 250 g. obtained from a random-bred strain of Wistar rat were used in these experiments.

Production of infection

Renal infection was produced by the direct inoculation of *E. coli* 075 into the kidney (Chapter 2).

Immunosuppressive therapy

6-mercaptopurine (6MP). Two dosages were used in these experiments: 10 mg./kg./day and 20 mg./kg./day. A solution of 6MP was prepared immediately before injection by dissolving the powder in 0.1 NaOH with the aid of gentle heat. This preparation was then diluted with physiologically normal saline so that the appropriate dose of 6MP was administered in a volume of 1 ml. Administration of 6MP into the peritoneal cavity of lightly anaesthetized rats commenced two days
before the induction of pyelonephritis and continued daily for a total of 14 days.

Cyclophosphamide (Endoxan, Mead Johnson). Graded doses of 50, 75, 100 and 125 mg./kg. were given intraperitoneally. To reduce any toxic side-effects the drug was administered as a divided dose; half the amount was given two days after infection and the remainder two days later.

Thiamphenicol (Urfamycin Zambon SPA Milano-Vincenza, Italy. Also known as thiocymetin and dextrosulphenidol). A single dosage level of 250 mg./kg. of thiamphenicol was administered by intraperitoneal injection two days before induction of bacterial infection and continued for a total of 14 days. For administration the thiamphenicol powder was dissolved in 1 ml. of distilled water.

Collection of blood for serum antibody titres
See Chapter 8.

Determination of serum antibody titres
See Chapter 10.

Eradication of infection with antibiotic treatment
Renal infection was eliminated by combined intramuscular therapy with gentamicin (20 mg./kg./day) and carbenicillin (Pyopen) (150 mg./kg./day). Antibiotic therapy was given for ten days, starting one week after the initiation of infection. Eradication of infection from renal tissue was achieved with this regime in 85% of animals.

Histopathological changes
Studies of the histopathological changes following renal
infection and treatment with immunosuppressive agents were carried out and the data compared with infected but untreated animals and, in some experiments, animals which had been infected and treated with antibiotics. Serial histological sections were examined to determine quantitatively the extent of renal damage, the intensity of lymphocytic infiltration in the pyelonephritic lesion and to assess the proportion of lymphocytes in the cellular infiltrate. The effect of immunosuppression on the inflammatory polymorphonuclear infiltrate and the degree of fibrosis was also examined.

Haematological screening

Blood was collected from the tail into heparinized capillary tubes and diluted immediately in an isotonic diluent (Isoton). Samples were processed in a Coulter counter, Model S, and the total leucocyte count, haemoglobin, red blood cell count, packed cell volume and red blood cell indices determined. A blood film for differential leucocyte count, and red blood cell morphology was also examined.

RESULTS

6-mercaptopurine

Pyelonephritis was induced in four groups, each of 12 rats. A control group remained untreated while the other three groups were treated with 6MP 10 mg./kg./day, 6MP 20 mg./kg./day and gentamicin-carbenicillin respectively. The effect of 6MP administration (20 mg./kg.) over a 14-day period is shown in Figure 81. Total leucocyte numbers decreased over the 10 to 21 day period, but later returned to normal. Haemoglobin levels
FIGURE 81.
The effect of 6-mercaptopurine administration on haematological values. The results presented are the mean and standard deviation of the results from 10 animals treated with 20 mg./kg./day for a total of 14 days.
declined slowly but also recovered over the final ten days of the period studied. The differential leucocyte count was not affected by 6MP treatment.

Twenty-eight days after infection, the animals were sacrificed and the infected kidneys removed. After assessing the gross pathology, half the kidney was homogenized to determine the bacterial content and the remaining half fixed for histological examination. The effect of 6MP treatment on the gross and bacteriological course of the disease is shown in Figure 82. Gross scarring 28 days after infection differed little between the four groups and animals in which the infection had been eliminated with antibiotic therapy showed degrees of scarring similar to untreated pyelonephritic animals. Administration of 6MP likewise did not affect the degree of scarring.

The effect of 6MP on the level of circulating antibacterial antibody showed that both dosages of 6MP suppressed the peak response at seven days. Significant antibody titres, however, still occurred and reached 20% of the normal response representing a 50-fold increase over baseline values in the rats given 10 mg./kg. and a ten-fold increase in rats given the higher dosage. In contrast to the control animals where the rapid increase in antibody levels was followed by an equally rapid decline, antibody produced by the 6MP treated animals persisted (Figure 83).

Because of the experimental design, it was only possible to examine the renal histopathology at one time point, 28 days after infection. The results are summarized in Table 18. When the extent of renal damage in the 6MP and antibiotic
The effect of 6-mercaptopurine and antibiotic treatment on the bacteriology and gross pathology of experimental renal lesions. Animals were killed 28 days after the induction of pyelonephritis. Quantitation of the gross pathology was made on a 0-10 scale with each unit of the scale representing one-tenth of the surface of the kidney. The term "residual infection" was used to indicate foci of incompletely healed lesions within scarred areas (see Chapter 2).
The effect of 6-mercaptopurine on the titre of circulating antibody in animals with E. coli pyelonephritis. Each point is the mean titre of 10 animals.
TABLE 18. Histopathology of the cellular infiltrate in the kidney of pyelonephritic animals treated with 6-mercaptopurine and antibiotics compared with the histopathology of pyelonephritic but untreated animals.
<table>
<thead>
<tr>
<th></th>
<th>No. of animals</th>
<th>Degree of renal damage*</th>
<th>Degree of mononuclear infiltration*</th>
<th>% of infiltrate</th>
<th>Presence of polymorphs</th>
<th>Fibrosis*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control infection</td>
<td>12</td>
<td>7.6 +</td>
<td>6.2</td>
<td>67</td>
<td>1.8</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.4) †</td>
<td>(0.6)</td>
<td>(4.2)</td>
<td>(0.6)</td>
<td>(0.8)</td>
</tr>
<tr>
<td>6MP 10 mg./kg.</td>
<td>11</td>
<td>7.6 +</td>
<td>5.6</td>
<td>48</td>
<td>3.0</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.6) †</td>
<td>(0.6)</td>
<td>(7.0)</td>
<td>(0.6)</td>
<td>(0.6)</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.1</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>6MP 20 mg./kg.</td>
<td>8</td>
<td>6.6 +</td>
<td>5.4</td>
<td>45</td>
<td>2.4</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.6) †</td>
<td>(0.6)</td>
<td>(5.3)</td>
<td>(0.8)</td>
<td>(3.0)</td>
</tr>
<tr>
<td></td>
<td>0.02 †</td>
<td>NS</td>
<td>NS</td>
<td>0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Gentamicin-Carbenicillin treated</td>
<td>7</td>
<td>5.2 +</td>
<td>5.0</td>
<td>48</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.0) †</td>
<td>(0.6)</td>
<td>(5.5)</td>
<td>(0.4)</td>
<td>(0.6)</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.02</td>
<td>NS</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Quantitative 0-10 scale used to make a comparative assessment of the cellular infiltrate and degree of renal damage (see Chapter 2).
† Mean for the group.
‡ Standard error.
¢ Degree of significance when compared to control animals (infected not treated) using the Wilcoxon sum of ranks analysis.
treated groups was compared with the control untreated infec-
tions, there were no consistent differences although animals
treated with the higher dose of 6MP did show a greater degree
of renal damage. No affect on the intensity of the lymphocytic
infiltrate was found, but a fall occurred in the ratio of
lymphocytes to other infiltrating cells. Again, 6MP at the
higher dose caused a relative decrease in the percentage of
lymphocytes in the cellular infiltrate.

Cyclophosphamid

Twenty animals were challenged on the same day and divided
into five groups each with four animals. This protocol was
repeated three times over the period of a week to give a total
of 12 animals per group. An additional animal was included in
each group as a precaution against postoperative deaths, and
accounts for 13 animals being analysed in two of the groups.
In the case of the group treated with 100 mg./kg. of cyclo-
phosphamid, an additional group of animals and their controls
was also studied. Four of the groups were subsequently treated
with cyclophosphamid with doses of 50, 75, 100 and 125 mg./kg.
respectively. The remaining group was untreated. Cyclophos-
phamid was administered two and four days after infection in a
divided dose and the animals sacrificed 28 days after the in-
duction of pyelonephritis. Administration of cyclophosphamid
(100 mg./kg.) caused a transitory decrease in the total number
of lymphocytes in the peripheral blood and an absolute lympho-
penia. The total and differential leucocyte count later
returned to normal. Haemoglobin levels followed a similar
pattern but the red blood cell numbers were not affected
The ability of cyclophosphamide to suppress the humoral immune response to renal infection was effectively demonstrated in these experiments. All dosages of cyclophosphamide tested suppressed the antibacterial antibody response. The maximum antibody titre observed during the first ten days following infection was less than 1% of the titre of the control animals (Figure 85). From the fourteenth day of infection all groups showed a slow increase in serum antibody which was dose related. The effect of treatment with cyclophosphamide on the gross changes and bacteriological course of the infection are shown in Figure 86. There were no significant differences when the gross scarring of cyclophosphamide treated animals were compared with the control animals ($P > 0.1$ for all treated groups). In some of the scarred areas, foci of incompletely healed lesions which we have called "residual infection" were found. There were no statistically significant differences between the immunosuppressed and the control groups but in 3 out of 12 and 3 out of 20 animals treated with 125 and 100 mg per kg, respectively, resolution of the lesions appears to be delayed.

The dramatic effect of cyclophosphamide in reducing the number of bacteria in the kidney was unexpected. All groups of animals treated with cyclophosphamide were found to have significantly fewer bacteria present in their kidneys when compared with the number of bacteria in the kidneys of untreated animals 28 days after infection. ($P = < 0.001$ for all cyclophosphamide treated groups). Many of the kidneys from the cyclophosphamide treated animals were sterile, and when
FIGURE 84.

The effect of cyclophosphamide administration on haematological values.
The results presented are the mean and standard deviation of the values from
6 animals treated with 100 mg./kg. of cyclophosphamide administered in a split
dose 2 and 4 days after the experiment commenced.
FIGURE 85.

The effect of cyclophosphamide on the titre of circulating antibody in animals with pyelonephritis. Each point is the mean titre of 10 animals.
FIGURE 86.

The effect of cyclophosphamide treatment on the bacteriology and gross pathology of experimental renal infection induced 28 days previously. Quantitation of the gross pathology was made on a 0-10 scale with each unit of the scale representing one-tenth of the surface of the kidney. The term "residual infection" was used to indicate foci of incompletely healed lesions within scarred areas (see Chapter 2).
individual kidneys were examined, it was sometimes found that kidneys showing histologically active lesions were also sterile. One explanation for the favourable effect of treatment with cyclophosphamid on the elimination of bacteria from the pyelonephritic kidney, was that the immunosuppressive agent also acted as an antibiotic. This seemed improbable in view of the timing and the comparatively small doses of drug administered; nevertheless, experiments were carried out to determine whether cyclophosphamide had any direct antibacterial activity. Three groups of 12 animals with induced renal infections were treated with 75 mg./kg. of cyclophosphamide two and four days after infection and sacrificed seven days after challenge; a second group was treated two and four days after infection with gentamicin which had been shown to be effective at eliminating renal infection. A third control group was infected but untreated. When the number of bacteria remaining in the kidney was determined, there was no evidence to suggest an antimicrobial effect with cyclophosphamide treatment (Figure 87).

Histopathological changes in animals treated with doses of cyclophosphamide from 50 mg./kg. to 125 mg./kg. were compared with infected but untreated animals (Table 19). Despite very low levels of circulating antibody the extent of the renal lesions in cyclophosphamide treated animals was not significantly greater than in control animals. Differences in the intensity of the round cell infiltrate were found at the two higher doses of cyclophosphamide and similar changes were also seen in a reduced proportion of lymphocytes in
FIGURE 87.

Failure of cyclophosphamide administration to exert an antimicrobial effect. Cyclophosphamide (75 mg./kg.) and gentamicin (20 mg./kg.) were administered 2 and 4 days after the induction of pyelonephritis. All animals were sacrificed 7 days after challenge.
TABLE 19. Histopathology of the cellular infiltrate in the kidney of pyelonephritic animals treated with cyclophosphamide
<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Degree of renal damage*</th>
<th>Degree of mononuclear infiltration*</th>
<th>Degree of infiltration</th>
<th>Presence of polymorphs</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control infection</td>
<td>11</td>
<td>6.8 + (1.0) ‡</td>
<td>6.2 (0.6)</td>
<td>67 (4.2) 33 (4.2)</td>
<td>5.2 (0.6) 3.0 (0.6) 5.2 (0.2)</td>
</tr>
<tr>
<td>Treated with Cyclophosphamide 125 mg./kg.</td>
<td>10</td>
<td>7.4 + (0.6) ‡</td>
<td>4.6 (0.4)</td>
<td>49 (5.2) 51 (5.2)</td>
<td>4.2 (1.0) 2.6 (1.0) 5.2 (0.4)</td>
</tr>
<tr>
<td>100 mg./kg.</td>
<td>11</td>
<td>7.2 + (0.4) ‡</td>
<td>4.1 (0.2)</td>
<td>43 (5.1) 47 (5.1)</td>
<td>2.8 (0.8) 1.8 (0.8) 6.2 (0.4)</td>
</tr>
<tr>
<td>75 mg./kg.</td>
<td>9</td>
<td>8.0 + (0.6) ‡</td>
<td>5.2 (0.8)</td>
<td>55 (6.2) 46 (6.2)</td>
<td>3.6 (0.6) 1.1 (0.6) 6.2 (0.6)</td>
</tr>
<tr>
<td>50 mg./kg.</td>
<td>9</td>
<td>6.3 + (1.2) ‡</td>
<td>5.0 (0.6)</td>
<td>54 (6.4) 45 (6.4)</td>
<td>4.0 (1.0) 1.8 (1.0) 5.5 (0.4)</td>
</tr>
</tbody>
</table>

* Quantitative 0-10 scale used to make a comparative assessment of the cellular infiltrate and degree of renal damage.
+ Mean for the group.
† Standard error.
‡ Degree of significance when compared to control animals (infected not treated), using the Wilcoxon sum of ranks analysis.
inflammatory infiltrate. Administration of cyclophosphamide, however, had little effect on the interstitial polymorphonuclear infiltrate or the degree of fibrosis.

**Thiamphenicol**

Pyelonephritis was induced in two groups of 12 animals, one of which was treated with 250 mg./kg./day of thiamphenicol two days before challenge, and subsequently daily for a total of 14 days. This dosage had a minimal effect on the haematological values of peripheral blood, although an increase in the numbers of leucocytes occurred (Figure 88). Thiamphenicol treatment resulted in similar changes in serum antibody levels to those observed following 6MP administration (Figure 39). A delay in the rise of circulating antibody was found in the thiamphenicol treated animals after which they responded with a sharp increase in antibody production which paralleled the response in normal animals but at a lower level. At sacrifice 28 days after challenge, although the bacteriological data were comparable, a highly significant difference in the degree of gross renal damage was apparent when the thiamphenicol treated and control rats were compared (Figure 90).

A likely explanation for the favourable effects of thiamphenicol on the pyelonephritic process is that the effects of the antibiotic properties of thiamphenicol rather than its immunosuppressive activity were being observed. To investigate this further, pyelonephritis was induced in three groups of 12 animals. One group was treated with thiamphenicol, 250 mg./kg./day, two days before infection and subsequently daily for a total of seven days. Two days later, the animals were
The effect of thiamphenicol administration on haematological values.

The results presented are the mean and standard deviation from 10 animals treated with 250 mg./kg./day for 14 days.
FIGURE 89.

The effect of thiamphenicol treatment (250 mg./kg. for 14 days) on the titre of circulating antibody in animals with E.coli pyelonephritis. Each point is the mean titre of 10 animals.
The effect of thiamphenicol treatment (250 mg./kg. for 14 days) on the bacteriology and gross pathology of experimental renal lesions. Animals were killed 28 days after the induction of pyelonephritis. Quantitation of the gross pathology was made on a 0-10 scale with each unit of the scale representing one-tenth of the surface of the kidney (see Chapter 2).
sacrificed and the gross pathology and bacteriology determined. A second group was treated with gentamicin, 10 mg./kg., according to the same schedule as the thiamphenicol treated animals, and the third group served as control. The results are shown in Figure 91. Administration of thiamphenicol and gentamicin prior to challenge prevented the development of pathologic lesions, but did not affect the establishment of infection in kidneys. Large numbers of bacteria were present in the kidneys from all groups, although the bacterial counts in both the treated groups were generally lower than the control group. These two experiments have shown that, although infection occurred in the kidneys of both thiamphenicol treated and non-treated control animals, pathological changes were not established to the same degree in the thiamphenicol treated animals. It is likely that the antibiotic effect of continued administration of the thiamphenicol also led to the suppression of the gross changes found 30 days after challenge. This was confirmed in a final experiment where the course of infection in a further three groups of 12 animals was investigated. One group was treated with thiamphenicol, 250 mg./kg./day, two days before infection and subsequently daily for a total of 14 days. A second group was treated with gentamicin, 10 mg./kg./day according to the same schedule while a further group served as a control. Twenty-eight days after challenge, in contrast to the control group, there were minimal changes in the kidneys from all animals treated with thiamphenicol and gentamicin.
The effect of thiamphenicol (250 mg./kg./day) and gentamicin (10 mg./kg./day) on the establishment of pyelonephritis. Treatment commenced 2 days before challenge and was continued for a further 5 days. 7 days after challenge, the animals were killed and the bacteriologic and pathologic changes determined. Quantitation of the gross pathology was made on a 0-10 scale, with each unit of the scale representing one-tenth of the surface of the kidney (see Chapter 2).
DISCUSSION

This study was undertaken to determine the role of the immune response in renal infection by manipulating the immunologic responsiveness of the host with immunosuppressive agents. Treatment with 6MP depressed the humoral immune response, but did not have a significant adverse effect on the course of renal infection. Administration of thiamphenicol was able to prevent the development of pathologic lesions, but this was attributed to the antibacterial spectrum of thiamphenicol rather than its immunosuppressive activity. Treatment with cyclophosphamide abolished the ability of the host to produce antibacterial antibody but unexpectedly enhanced the capacity of the host to eliminate the invading organism.

The biology of the immune response to renal infection has been studied by many investigators in an attempt to determine diagnostic features (Hanson & Winberg, 1966; Bremner et al., 1969), host-protective functions (Brooks, Lyons & Braude, 1974) and the role of autoimmunity as a factor in immunologically mediated renal disease (Montgomerie, Kalmanson & Guze, 1969; Miller et al., 1970).

The immunosuppressive agents used in the present studies were selected on the basis of their differing pharmacologic properties so as to inhibit specific steps in the immune response. 6MP by inhibiting the nucleic acid metabolism of dividing cells is cell-cycle specific, whereas cyclophosphamide, a non-cycle dependent drug, is active against intermitotic as well as dividing cells. Thiamphenicol, an analogue of chloramphenicol, was of interest because of its dual anti-
bacterial and immunosuppressive properties. The immunosuppressive effects of thiamphenicol on active antibody production (Weisberger & Daniel, 1969) and graft rejection (Simpson & Richmond, 1974; Linehan et al., 1969) have previously been studied and in addition it has been widely used as an antibiotic.

There is evidence from other studies that treatment with 6MP may alter the course of an infectious disease. Foster & Moll (1968) were able to affect favourably both the pathological and clinical course of Marek's disease where the primary damage is initiated by an infectious agent, but the subsequent pathological lesions have an immunological basis. Other investigators have noted that treatment with 6MP had a significant effect on an acute inflammatory lesion and virtually eliminated the mobilization of mononuclear cells into the acute lesion (Page, Condie & Good, 1962). The failure of 6MP to alter significantly the pathology and course of renal infection, despite a demonstrable effect on the humoral immune response, suggests that even a diminished humoral response is capable of containing the infection, or that non-specific immune factors may be more important. The failure we observed of 6MP treatment to affect the degree of cellular infiltrate in the pyelonephritic kidney of most animals is compatible with other reports where 6MP had only a minor effect on the cellular infiltrate in chronic inflammatory lesions (Stevens & Willoughby, 1969).

Less is known about the immunosuppressive activity of thiamphenicol although one recent report suggested that its
immunosuppressive activity results from anti-proliferative action on cells with an increased mitotic activity (Della Bella et al., 1973). In the current experiments, thiamphenicol appeared to delay the humoral immune response to infection and suppress the peak response although a comparison with infected untreated control animals was complicated by the inability of *E. coli* to establish pathological lesions in the thiamphenicol treated animals. Renal infection, however, was initiated even during treatment with thiamphenicol and gentamicin and it was a matter of considerable interest that pathological lesions did not develop as a result of this infection. Consideration of the humoral immune response in relation to the pathological and bacteriological data is also revealing. Treatment of animals with thiamphenicol at the time of challenge largely prevented the establishment of pathological lesions in the kidney, but all animals still responded to this challenge with a marked humoral immune response. The present experiments, however, were not able to distinguish an immune response occurring as a result of renal infection from the response of the host to the transient bacteraemia which may have occurred when *E. coli* were inoculated directly into the kidney. Nonetheless, the results do emphasize the difficulty of using serum antibody responses as an index of pathological changes in the kidney.

The effect of cyclophosphamide on the immune response to renal infection and the bacteriological course of the disease was striking. Administration of cyclophosphamide (50 to 125 mg./kg.) effectively suppressed the immune response so that circulating antibody levels were reduced to less than 2% of normal during the peak response. The ability of
cyclophosphamide to reduce effectively bacterial persistence in the kidney, and in some cases to eradicate the infection, was surprising. With this model of experimentally induced infection, the capacity of the infecting organisms to persist in the kidney has been consistently demonstrated. An inverse relationship was found between the capacity of the host to mount an immune response and bacterial persistence in the kidney so that animals that failed to synthesize antibacterial antibody were able to eliminate the causative organism more effectively than were untreated animals. A possibility that the effect of cyclophosphamide was due to antibacterial activity was considered but discounted after cyclophosphamide treatment failed to show any antibacterial effect. Even gentamicin, which was known from previous experiments to be capable of eliminating renal infection when an adequate course of treatment was given, could not eliminate infection when given according to the same schedule as the cyclophosphamide. It was therefore most unlikely that cyclophosphamide administration could have resulted in the elimination of infection as a result of any antimicrobial properties. This conclusion is supported by a recent study where urine samples of Rhesus monkeys treated with cyclophosphamide were examined for antimicrobial activity. No antibacterial activity against seven bacterial strains commonly causing urinary tract infections was found (Primack, 1973). In view of the recently demonstrated effect of cyclophosphamide metabolites on the function of the distal renal tubule, a further consideration remains that the administration of cyclophosphamide may have altered the medullary environment and subsequently the course of infection (De Fronzo et al., 1974). An increase
in urinary osmolality occurred 4 to 12 hours after cyclophosphamide administration and persisted for 20 to 24 hours. The effect of the medullary osmolality on the course of renal infection has been argued at length, but in view of the short half life (6 to 7 hours) and the transient effect of cyclophosphamide on the medullary environment, it is unlikely that physiological changes induced by cyclophosphamide metabolites can explain the results found in the present experiments.

These observations are best explained by the blocking of the phenomena of immunological enhancement, or immune facilitation as suggested by Voisin when enhancement occurs in situations other than tumour immunology (Voisin, 1971). The mechanism by which the immune response enhances tumour growth is thought to involve humoral antibody and has been attributed to the ability of the antibody to protect tumour cells against destruction by a cell-mediated immune response. These and related observations are, we believe, relevant to the immunobiology of pyelonephritis where persistence of infection is a characteristic feature of both experimental infection and the disease in man, despite a seemingly adequate immune response. The host-parasite relationship in this situation has been explored and the term "acquiescent infection" used to describe the relationship between host and parasite in chronic renal infection (Chapter 8). Immunological enhancement should be considered as an explanatory mechanism for the phenomena, although there is no experimental evidence for this in the literature of infectious disease (Voisin, 1971). Medici (1972) and Voisin (1971), however, have both suggested that enhancement may be
an immunoprotective mechanism favouring the persistence of pathogenic micro-organisms.

Further support for the role of enhancing antibody in infectious disease can be found in several investigations of host defence mechanisms. Williams et al. (1969) in a study of patients with chronic osteomyelitis, described a protein in the immunoglobulin fraction which, when allowed to react with bacteria, was able to block the action of a normally active phagocytic system. Taylor (1972) has described an immunoglobulin isolated from the serum of patients with pyelonephritis which protects Gram negative organisms from the bactericidal activity of serum, and Waisbren & Brown (1966) have also described a factor in the serum of patients with chronic renal infection that can inhibit normal bactericidal mechanisms.

One recent study has shown that cyclophosphamide may similarly alter the course of a virus (Singer, Noguchi & Kirschstein, 1972) infection. The mortality of an influenza virus infection in mice was decreased in this experiment from 65% to 22% during the first week by treatment with cyclophosphamide despite a decrease in circulating antiviral antibody. This favourable effect of cyclophosphamide therapy was attributed to a decrease in the cellular infiltrate of the lungs and not to increased viral elimination.

The possibility that immunoglobulins produced locally within the kidney (Lehmann et al., 1968; Miller & North, 1971; Miller et al., 1971) may become bound to the infecting organism and act in an enhancing role, now seems a distinct possibility following the demonstration by Thomas et al. (1974) and Jones et al. (1974) of antibody coated bacteria in the urine of
patients with renal infection. Local antibody may also be involved in the formation of immune complexes which could act in an enhancing role by blocking the activity of mononuclear phagocyte cells. Both viable and non-viable bacterial antigen has been shown to persist in renal infection (Cotran, 1963) and could readily form complexes with locally produced antibody. Immunoglobulin receptors have been demonstrated on the surface of mononuclear phagocyte cells (Arend & Mannik, 1972) and if these receptors are involved in the initial contact with opsonized bacterial cells, then saturation of receptor sites with antibody could be the mechanism contributing to immunological enhancement.

Finally, antibody to non-host-protective antigens of *E. coli* could be involved in immune complex formation or direct immune enhancement. The antigenic mosaic of *E. coli* has already been described (Holmgren et al., 1969) and differences in the capacity of antibodies directed at major antigens of *E. coli* to provide protection from reinfection have been found (Ahlstedt, Holmgren & Hanson, 1972). The role of non-protective antibodies in the immune response to infectious disease has yet to be defined so that their function in immunological enhancement remains uncertain.
The basis of this study has been the investigation of a clinical problem and throughout these experiments, clinical aspects have had a bearing on the nature of the investigation. In the section that follows, several diagnostic and therapeutic problems have been investigated using experimental animals and, in the final two chapters, material obtained from clinical sources in New Zealand and Australia. In the investigation of the relationship between the immunogenicity of the infecting organism and the host's immune response, the clinical work was carried out in Melbourne by Drs. Fairley and Whitworth.
CHAPTER 15

ESTIMATION OF THE IMMUNE RESPONSE TO BACTERIAL ANTIGEN

USING A HAEMAGGLUTINATING AUTOANALYSER
INTRODUCTION

Although a number of advances have been made in the sensitivity with which serum antibody levels can be determined, traditional and convenient methods using serial dilutions of serum are still commonly used in routine analysis and research investigations. Methods using doubling dilutions of serum, however, suffer from the major disadvantage that the titration end point is limited to one of the dilution values and intermediate values cannot be obtained.

In this chapter the adaption of an autoanalyser system for the measurement of antibacterial antibody is described. The objective was to have available a method for the quantitation of small increments in serum antibody levels and for it to be sufficiently sensitive to allow the detection of the small amount of antibacterial antibody which is thought to be present in the urine during renal infection. The method was found to be reproducible, show increased sensitivity compared with manual methods and to be capable of determining antibody titres on a continuous scale.

MATERIALS AND METHODS

Equipment

A Technicon autoanalyser manifold (Figure 92) similar to that described by Rosenfield & Haber (1966) was used with reagents added at the optimal concentrations and flow rates shown in Table 20. Sera to be tested were automatically sampled sequentially and introduced, together with reagents, through a
FIGURE 92.

Flow diagram of the Technicon autoanalyser used for the determination of antibacterial antibody.
TABLE 20. Optimal concentrations and flow rates for the reagents used in the automated determination of antibacterial antibody.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Flow rate (ml/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline/Tween</td>
<td>.02% Tween 80 in 0.98% saline</td>
<td>1.2</td>
</tr>
<tr>
<td>PVP</td>
<td>1.5% in 1.1% saline containing 0.02% Tween</td>
<td>0.1</td>
</tr>
<tr>
<td>Sampler wash</td>
<td>0.5% bovine albumin in 0.98% saline</td>
<td>1.6</td>
</tr>
<tr>
<td>Triton</td>
<td>0.6% Triton X 100 in distilled water</td>
<td>1.2</td>
</tr>
<tr>
<td>Sample</td>
<td>Serum diluted in 0.5% bovine albumin in 0.98% saline</td>
<td>0.1</td>
</tr>
<tr>
<td>Sensitized SRBC</td>
<td>4% suspension in saline containing 0.5% ficoll</td>
<td>0.1</td>
</tr>
</tbody>
</table>
constant speed pump into the reaction manifold. The suspension of erythrocytes sensitized with bacterial endotoxin was sampled coincidentally with the serum samples. The reactants were then mixed in a series of glass coils, before rouleaux formation, induced with polyvinylpyrrolidone (PVP), was dispersed by the addition of a saline-tween solution. Agglutinates were then allowed to settle in two horizontally placed settling coils and were subsequently removed. The remaining cells were lysed in a solution of Triton X 100, and the resultant haemoglobin solution passed through a continuous flow colorimeter where the optical density was automatically recorded. In this system, the concentration of the final haemoglobin solution was inversely proportional to the amount of antibody present.

Endotoxin preparation
Boivin-type endotoxin was prepared by tri-chloracetic acid extraction of cultures of Escherichia coli 075 and Escherichia coli 08, essentially as described by Webster et al. (1955). Details of the activation of the endotoxin have been given in Chapter 10.

Preparation of erythrocyte suspension sensitized with bacterial antigen
Blood was collected from random sheep into Alsevers solution and stored at 4°C. for seven days. The sheep red blood cells (SRBC) were washed three times in 0.9% saline. One volume of washed, packed erythrocytes was mixed with five volumes of 075 endotoxin and 45 volumes of PBS, or six volumes of 08 endotoxin and nine volumes of PBS, and incubated at 37°C. for one hour. The endotoxin-sensitised cells were then washed five times with
PBS. One volume of packed sensitized erythrocytes was added to four volumes of a solution of 0.5% bromelin in saline and incubated at 37°C for 15 minutes. The cells were then washed three times with PBS and finally prepared as a 4% suspension in saline containing 0.5% Ficoll. The suspension was standardized colorimetrically.

**Standard antibody preparation**

Antibody to *E. coli* 075 was raised in rabbits by the repeated intramuscular injection of heat killed antigen. Six weeks after the commencement of immunization, the animals were bled and the serum stored at -20°C in small aliquots until used. With each batch of analyses a new aliquot was thawed. Six dilutions from 1:500 to 1:7,500 were prepared in 0.5% bovine-albumin-saline and processed to determine the titre of the standard for that group of samples.

**Titration of antibacterial antibody using the autoanalyser**

Appropriate dilutions of serum in 0.5% bovine-albumin-saline were sampled at the rate of 20 per hour. The most suitable dilution range for each serum was selected after a preliminary screening of dilutions between 1:10 and 1:10,000. Corrected optical density values (i.e. optical density of baseline-optical density of test) were plotted against the serum dilution and subjected to a least squares analysis. The straight line produced was extrapolated to the Y axis to obtain the titration value. Titration values were corrected for day to day machine variation using the titration value for the standard antiserum obtained in the batch analysis and which was compared with the established titration value for the
standard. The recorder chart from a typical analysis is shown in Figure 93.

**Manual method**

Serum antibody levels were determined using the passive haemagglutination procedure, described in Chapter 10.

**Reduction of antibody activity with 2-mercaptoethanol**

Confirmation was sought that the automated procedure was capable of determining 2-mercaptoethanol sensitive and resistant antibody. A strain of Bagg mice that responded to SRBC immunization, initially with the production of IgM plaque-forming cells, and later IgG plaque-forming cells, were injected intraperitoneally with 0.25 ml. of a 10% SRBC suspension. Serum samples from mice were collected by cardiac puncture 3, 5 and 8 days after the initial injection, pooled, and stored at -20°C until tested. 2-mercaptoethanol treatment of serum was carried out by adding 50 μl. of serum to 200 μl. of 0.25M 2-mercaptoethanol and incubating the preparation at 37°C for one hour. Sera treated in this way were tested in the autoanalyser within six hours of treatment.

**Antibody to E.coli**

Fifteen random bred Wistar male rats were immunized with a single intramuscular injection of $1.0 \times 10^9$ heat killed E.coli 075. Serum samples were obtained on the day of immunization and 3, 7, 14 and 21 days after immunization. The antibody levels were estimated in the autoanalyser and by manual methods. Serum samples were tested against SRBC sensitized with endotoxin from E.coli 075 and also with SRBC sensitized...
FIGURE 93.

A tracing taken from the recorder chart of a typical analysis. When the optical densities were subjected to least squares analysis, a final titre of 2,030 was derived for this serum sample.
with *E. coli* 08 endotoxin to determine the specificity of the analysis.

**RESULTS**

Specificity and reproducibility of the procedure

The specificity of the immune response to *E. coli* 075 killed antigen was confirmed in the autoanalyser by testing serum samples against SRBC sensitized with endotoxin from *E. coli* 08 as well as from *E. coli* 075. The antibody response of the 15 animals immunized with killed *E. coli* 075 antigen is shown in Figure 94. A variable but consistent response to immunization with *E. coli* 075 killed antigen was observed. Eight animals also showed an increase in serum antibody titre against the 08 antigen. The titre increases followed a similar time course to the immune response against the immunizing antigen and were between 10 and 20% of the titres against the 075 antigen.

The reproducibility of the analysis was confirmed by comparing the results from seven successive batches of tests using the standard 075 and 08 antisera (Table 21). A coefficient of variation of 8.6% was obtained for the seven analyses of the anti 075 serum and demonstrates the high degree of reproducibility obtained using the autoanalyser.

Determination of the immune response to immunization with bacterial antigen using the autoanalyser

A characteristic peak antibody titre was found seven days after antigen challenge which declined rapidly over the following 14 days. A comparison of the results obtained from
FIGURE 94.

Serum antibody titres (mean ± SD) for 15 animals immunized with $1 \times 10^9$ E.coli 075. Serum samples were tested against SRBC sensitized with endotoxin from E.coli 075 and 08 to confirm the specificity of the method.
TITER USING 075 SENSITIZED SRBC
TITER USING 08 SENSITIZED SRBC.

SERUM ANTIBODY TITER

DAYS AFTER IMMUNIZATION
TABLE 21. Reproducibility of the automated procedure. Results of seven determinations of the standard anti-075 antisera and four of the standard anti-08 antiserum which were included in consecutive batches of routine analyses.
<table>
<thead>
<tr>
<th>Anti-075 standard antiserum</th>
<th>Anti-08 standard antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 4022</td>
<td>44914</td>
</tr>
<tr>
<td>2. 7441</td>
<td>47380</td>
</tr>
<tr>
<td>3. 8249</td>
<td>37306</td>
</tr>
<tr>
<td>4. 8638</td>
<td>38654</td>
</tr>
<tr>
<td>5. 9541</td>
<td></td>
</tr>
<tr>
<td>6. 9341</td>
<td></td>
</tr>
<tr>
<td>7. 8419</td>
<td></td>
</tr>
</tbody>
</table>

Mean: 8522  
S.D.: 733  
Coef. of variation: 8.6%  

Mean: 41164  
S.D.: 3746  
Coef. of variation: 9.0%
the automated and manual analyses has been made in Table 22. Although the profile of the immune response was similar when determined by manual or automated methods, the automated method was considerably more sensitive but a wide range in sensitivity was found.

2-mercaptoethanol treatment of serum
A mean 91% of the anti-SRBC antibody activity of serum samples from six mice taken three days after immunization with SRBC was inactivated by treatment with 2-mercaptoethanol. Two days later a figure of 83% was obtained and eight days after immunization only 37% of the serum antibody activity was 2-mercaptoethanol sensitive. This reflected the increased proportion of IgG (2-mercaptoethanol resistant) antibody being produced and confirmed the ability of the automated method to detect 2-mercaptoethanol sensitive and resistant antibody.

DISCUSSION
The high degree of accuracy and reproducibility using the autoanalyser equipment to measure haemagglutinating antibodies is generally accepted and the apparatus is in routine use in many laboratories. The present results using bacterial antigens attached to red cells has shown that the haemagglutinating autoanalyser could also be used to measure antibacterial antibody.

The simultaneous sampling of both test sera and cell suspension combined with the pre-analyser enzyme treatment of sensitized cells contributed substantially to the accuracy of the method. The reagent concentrations tabulated in Table 20
TABLE 22. Measurement by automated (A) and manual (M) methods of titres of antibody in sera of rats immunized with $10^9$ heat-killed Escherichia coli 075.
<table>
<thead>
<tr>
<th>Rat no.</th>
<th>Baseline</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>A</td>
<td>M</td>
<td>A</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>105</td>
<td>128</td>
<td>610</td>
<td>1024</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>32</td>
<td>305</td>
<td>256</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>64</td>
<td>307</td>
<td>2048</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>256</td>
<td>1007</td>
<td>512</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>168</td>
<td>32</td>
<td>814*</td>
<td>1024</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>0</td>
<td>4</td>
<td>0*</td>
<td>32</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>108</td>
<td>64</td>
<td>775</td>
<td>256</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
<td>512</td>
<td>1293</td>
<td>512</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>0</td>
<td>16</td>
<td>89</td>
<td>256</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>0</td>
<td>16</td>
<td>161</td>
<td>512</td>
</tr>
<tr>
<td>11</td>
<td>8</td>
<td>0</td>
<td>128</td>
<td>402</td>
<td>512</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>95</td>
<td>256</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>135</td>
<td>512</td>
<td>688</td>
<td>2048</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>75*</td>
<td>16</td>
</tr>
</tbody>
</table>

Average ratio (analyzer:manual)  5.6  7.3  11.0  13.4
(0.9)† (1.1) (2.1) (2.7)

* Individual analyses with widely divergent ratios that were not included in the calculation of the average analyzer-to-manual ratio.
† Standard error.
were determined experimentally and represent the optimum concentrations for this analysis. Minor variations were observed in the ability of different erythrocyte samples to be sensitized by bacterial antigens but these did not usually result in any practical difficulties.

The value of the automated analysis using a continuous scale is evident when small increments in antibody concentration are being sought (see Chapter 16). This is particularly important in sequential studies, and in Chapter 8 continuous analysis was used to advantage to determine the effect of activation of renal infection on the level of circulating antibody in individual animals with pyelonephritis.
CHAPTER 16

DIAGNOSTIC PARAMETERS IN EXPERIMENTAL RENAL INFECTION
Urinary tract infections are among the most common infections encountered in general medical practice. The clinical symptoms may be misleading, however, and as a result, considerable effort has gone into developing laboratory diagnostic tests. This has led to a proliferation of analyses, many of which have merit, but a simple and accurate diagnostic test has yet to be devised. One of the problems has been that the site of infection or even the presence of infection has been difficult to define so that it has not always been possible to relate the results of many of the studies to accurate clinical information. Experimental models, on the other hand, have the advantage that both the site of infection and the causative organism are known and allow the laboratory data to be correlated directly with a definite clinical and pathological entity. In this study, experimentally induced renal infections were used to investigate the use of urinary antibody, rheumatoid factors, histochemical examination of renal tissue and serum antibody levels as diagnostic aids.

MATERIALS AND METHODS

Animal strains
Female rats weighing 225 to 250 g. obtained from a random bred strain of Wistar rat, and N.Z. white rabbits were used in these experiments.

Production of renal infection
(i) Unilateral pyelonephritis in the rat was produced by the
direct inoculation of *E. coli* 08 into the surgically exposed kidney (Chapter 2).

(ii) A similar procedure was used to induce pyelonephritis in the rabbit using a 26-gauge needle and a total of $5 \times 10^6$ organisms injected into the kidney at three sites.

(iii) Retrograde pyelonephritis was induced by the implantation of a small glass capillary containing bacterial inoculum in agar into the dome of the surgically exposed bladder (Chapter 6).

**Determination of serum antibody response**
See Chapter 10.

**Measurement of urinary antibody**

(i) Collection and concentration of urine from rabbits with pyelonephritis.

A stainless steel funnel shaped tray was placed under a rabbit cage fitted with a stainless steel mesh bottom to retain any faecal pellets. Urine was collected over a period of 24 hours into a container held at $0^\circ$C. During the period of the collection, food was withdrawn but the rabbits did have access to water.

Urine samples were centrifuged at 5,000 r.p.m. for ten minutes to deposit any suspended material and the supernatant dialysed for 24 hours against distilled water. Concentration was carried out using 20 g. of powdered polyethylene glycol flakes held in dialysis tubing which was then suspended in the urine for 24 hours. Two changes were sufficient to reduce a 200 ml. volume of urine down to approximately 30 ml. and at this stage the final concentration was carried out in a 10 cm. x 2.5 cm.
- 211 -
tube with a finger-like extension in the bottom. Two grams of powdered polyethylene glycol flakes were held in a glass tube one end of which was sealed with a dialysis membrane and through which diffusion took place when this tube was inserted into the concentrating tube containing urine. The urine sample was stirred continually with a small bar magnet during the final concentration step which reduced the sample to a volume of 2 ml.

(ii) Quantitation of urinary antibody.
A Technicon autoanalyser was adapted to determine antibacterial antibody using the passive haemagglutination of endotoxin treated sheep-red-blood cells, and their subsequent lysis, as an indicator system. The method has been described in the previous chapter.

Histochemical examination
The normal and pyelonephritic kidneys from each animal were fixed in 10% neutral formalin for one week and serial 6 μ sections cut through the entire kidney after processing.
Sections were stained with haematoxylin and eosin and a series of connective tissue, glycoprotein and mucopolysaccharide specific stains, including the Masson-trichrome, colloidal iron, alcian blue/periodic-acid-Schiff (PAS), and alcian blue/aldehyde fuchsin (Masson, 1929; Mowry, 1963; Spicer, Horn & Leppi, 1967).

RESULTS
Rheumatoid factor in renal infection
Serum was taken from five rabbits at weekly intervals after initiating a unilateral renal infection with E.coli. All
serum samples were tested for rheumatoid factor using rabbit gamma-globulin as antigen in the Waaler-Rose test. Serum antibody levels were also determined on the same samples. The results are shown in Table 23. Although all animals showed an increase in serum antibody levels which was maintained for several weeks, significant levels of rheumatoid factor were not found in any of the sera tested.

**Urinary antibody in renal infection**

Urine collections were made from five rabbits at weekly intervals for the three weeks preceding the induction of unilateral pyelonephritis. Urine samples were then collected for 24 hours each week over a 35-day period during the course of pyelonephritis and the specific antibody titre of the concentrated urine determined in the autoanalyser. Antibody against the infecting organism was not found in any of the concentrated urines collected before the induction of pyelonephritis but subsequently it was possible to demonstrate antibody in the urine of all the pyelonephritic animals (Table 24).

**Histochemistry**

Kidneys from pyelonephritic animals were sectioned and stained to demonstrate selected histochemical features. Kidneys examined seven days after infection showed increased collagen deposition with thickening of the basement membrane around some of the tubules. The iron stain showed little additional organised polysaccharide although occasional interstitial deposits were seen with PAS - alcian blue stain. A general increase in mucopolysaccharide was found in the infected
TABLE 23. Serum antibacterial antibody and Rose-Waaler titres in rabbits with experimental pyelonephritis.
<p>| TABLE 24. Urinary antibody in renal infection |</p>
<table>
<thead>
<tr>
<th>Rabbit No:</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Titre of urinary antibody</strong></td>
<td><strong>days before renal infection</strong></td>
<td>21</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>days after renal infection</strong></td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>96</td>
<td>160</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>37</td>
<td>115</td>
<td>24</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>950</td>
<td>82</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>
regions with intensive staining of the nuclei in the renal
tubular cells.

The nature of the histiocyte accumulation in the infected
kidney was of particular interest. Aggregates of histiocytes
contained glycoprotein and polysaccharide granules which
stained intensely with PAS-alcian blue or with colloidal iron
and were only found in the pyelonephritic kidneys. The most
unique feature, however, was the formation of histiocyte
rosettes around the renal tubules in the pyelonephritic animals
which were not seen in other forms of renal trauma (Figures
95 and 96).

In more advanced lesions 30 days after infection, a consider-
able increase in the number of PAS-staining histiocytes were
found together with an increase in the amount of polysaccharide-
containing material in the tubular lumen.

Correlation of the serum antibody response with the
pathological data

Thirty-eight rats were immunized with $1 \times 10^7$ heat killed
E. coli 08 and 30 days later renal infection was established
by a retrograde challenge using a viable culture of E. coli 08.
The immune response to infection over the following 21 days
was then determined. This protocol was used to simulate the
conditions occurring in recurrent infections, where the host
has been previously exposed to the infecting organism. At the
completion of the experiment, kidneys were removed for histo-
logical and bacteriological examination and this data correla-
ted with the immune response to challenge. An increase of two
or more dilutions in the serum antibody levels over the
FIGURE 95.

Histological section of a kidney from a rat with pyelonephritis showing the tubules containing granules staining with periodic-acid-Schiff and alcian-blue histochemical stains.

FIGURE 96.

A rosette of histiocytes containing periodic-acid-Schiff (PAS) and alcian blue staining material which completely surround the renal tubule of a rat with active renal infection. PAS staining histiocytes may also be seen inside the lumen of the tubule. The staining characteristics suggest that much of the material staining inside the histiocytes is glycoprotein.
TABLE 25. Correlation of the serum antibody response with pathological data in renal infection.
Significant increase in antibody titre 28
  
  ( Positive pathological data 21
  ( Acquiescent infection 8
  Negative pathological data 7

No significant increase in antibody titre 10

  ( Positive pathological data 1
  ( Acquiescent infection 1
  Negative pathological data 9
baseline titre before retrograde challenge was accepted as a significant response to the infecting challenge.

Twenty-eight of the 38 rats did show a significant increase in antibody titre and 21 of these had positive pathological findings (Table 25). In eight of these the evidence for pathological involvement consisted of renal infection without any histopathological changes. This situation has previously been described as acquiescent renal infection in Chapter 8. Ten animals failed to register an increase in antibody titre and in nine of these, no pathological data was found. The remaining animal was found to have an acquiescent infection.

DISCUSSION

The problem of urinary tract infection and its many forms of clinical and pathological presentation has led to the introduction of a number of clinical and laboratory investigations which have been developed to provide a more accurate diagnosis (Fairley et al., 1967; Lampert & Berlyne, 1971; Mattenheimer, 1971; Jones, Smith & Sanford, 1974). In this study, experimentally induced renal infections were used to explore the possible contribution of a number of diagnostic parameters. The serum antibody response to challenge was of interest when the results of the immune response were collated with the pathological and bacteriological data from individual animals. Correlation between the increase in serum antibody levels and the pathological data was surprisingly good, but only if the acquiescent state is accepted as an entity (Chapter 8). Renal infection alone without pathological
changes did result in significant titre increases, whereas only one of the ten animals with no significant increase in serum antibody levels had evidence of infection.

The value that other investigators have attached to serum antibody titres as a diagnostic aid in clinical renal infection has varied enormously. While some authors have found a favourable correlation between renal infection and an increase in serum antibody levels (Percival, Brumfitt & De Louvois, 1964), others have not been able to confirm these results and believe this estimation to be of limited value (Fairley et al., 1971). Experimentally there has been a divergence of opinion although a correlation has been found in a number of investigations. To some extent this is to be expected and the present studies investigate the problem in the most favourable of circumstances.

Under these conditions, the aetiology of the disease is known, the immune status can be controlled, the causative organism is available, the site of infection is defined, pre-infection serum sampling is not a problem, and the collection of sequential post-infection samples does not present any difficulties. In clinical medicine each of these items presents as a variable or even an unknown factor so the poor correlation many investigators have reported between circulating antibody levels and the eventual diagnosis is understandable.

The present experiments do offer some encouragement in that serum antibody levels were associated with active infection in a high proportion of renal infections. The limitations which restrict the value of this simple analysis at the moment may, however, be overcome in the future as procedures for
determining the physicochemical characteristics of the specific antibody become more readily available.

The appearance of histiocyte rosette containing glycoprotein and polysaccharide material which were clustered around the renal tubules was particularly striking. Similar cells have been described in the renal interstitium of animals with active renal infection by Tan & Heptinstall (1969) and Cotran (1969) who both considered it likely that bacteria played some role in the aggregation of PAS positive histiocytes. Observation of the clustering of the histiocytes around the tubules has not been previously reported and was only seen in the kidneys of animals with pyelonephritis. When additional experiments were carried out using other forms of renal damage, histiocyte rosettes were not found. If similar rosettes can be found by the histochemical examination of renal tissue in man, their presence may be useful in implicating an infectious process in the pathogenesis of inflammatory lesions in the kidney.

An interest in demonstrating the possible association of rheumatoid factor with renal infection arose from earlier studies associating antibody to globulin with infections of known aetiology (Williams & Kunkel, 1962). Rheumatoid factors have also been found principally in the IgM fraction of the gamma-globulin and as the immune response to E.coli lipopolysaccharide has been shown to be predominantly IgM, it was anticipated that this too favoured the demonstration of rheumatoid factor. A rheumatoid factor-like substance has also been observed in rabbits immunised with E.coli antigen (Abruzzo & Christian, 1961), but in the present experiments, no
increase in the serum levels of rheumatoid factor was demonstrable when the serum from rabbits with pyelonephritis was tested over a five to seven week period.

These results are consistent with a recent study where the correlation between infections and serum antiglobulin factors similar to rheumatoid factor (serum agglutinators) was investigated. An increase in serum agglutinators was found with infections caused by Gram positive organisms, but Gram negative bacilli failed to stimulate a detectable response. In the same study, positive results for rheumatoid factors were associated with hyperglobulinaemia, but when sera from 102 patients were tested, individuals with infections rarely produced significant titres of macroglobulin antibody to globulin rheumatoid factor (Waller & Duma, 1972); on the evidence available, it seems that serum analysis for rheumatoid-like factors has little application for the diagnosis of renal infection.

Specific urinary antibody could be consistently demonstrated in the concentrated urine from rabbits with pyelonephritis when a sensitive method utilizing a haemagglutinating autoanalyser was used. A number of related studies have demonstrated the presence of antibody in the urine directed against bacteria, bacterial toxins, and phage particles (Merler et al., 1963; Hanson & Tan, 1965; Turner & Rowe, 1967) and IgG antibody activity against the somatic antigen of the infecting organism has been found in the urine from individuals with urinary tract infections (Vosti & Remington, 1968; Hanson et al., 1971). Patients with urinary tract infection have been shown to have an elevated level of secretory IgA in the urine (Burdon, 1970;
Kaufman, Katz & McInsoh, 1970; Uehling & Steihm, 1971) and this has recently been correlated with increased antibacterial activity (Jodal et al., 1974).

Considerable impetus has been given to this topic by a series of experimental studies which have attempted to establish whether antibody activity in the urine is derived from serum antibody or synthesized locally within the infected kidney and excreted in the urine. Local synthesis of IgG immunoglobulin as well as specific antibacterial antibody has been found in pyelonephritis (Lehmann et al., 1968) and in chronic healed focal infection of the kidney (Miller, Smith & Sanford, 1971). Related studies have confirmed these findings by demonstrating the presence of specific antibacterial antibody forming cells in the kidney throughout the course of pyelonephritis (Miller & North, 1971; Spencer & Fairhead, 1972), the eventual accumulation of locally synthesized antibody in the pyelonephritic kidney (Miller & North, 1973) and the contribution of local antibody synthesis by the bladder (Smith & Hand, 1972) to antibody in the urine.

Many of these studies have been qualitative rather than quantitative and have used research procedures to isolate and measure the antibody present. One of the objectives of the present study was to determine whether specific antibody could be detected in the urine in animals with pyelonephritis by using an autoanalyser adapted to detect antibacterial antibody. A simple concentration method using inexpensive materials was also introduced, and as a result, it was possible to quantitate the amount of specific antibody in the urine of animals with
experimentally induced renal infection. Consideration was
also given to the relationship between urinary antibody, histo-
pathological changes in the kidney suggestive of pyelonephritis
and the presence of active infection. The acquiescent state,
where renal infection is not associated with pathological
changes in the kidney but where the host does not seem capable
of eliminating the invading organism, has already been discussed
in Chapter 8. Some of the possible permutations of pathological
conditions and diagnostic data have been presented in Figure 95.
The assumption has been made that the investigator has informa-
tion from a renal biopsy, bacteriological culture of ureteric
urine and a quantitative measure of the amount of specific
antibody present in the urine. If the acquiescent state is
accepted, at least four common pathological conditions and
their diagnostic features can be recognised. One criticism
might be that undue emphasis has been placed on the demonstra-
tion of specific antibody in the urine. However, as has been
argued in this discussion and also shown experimentally, there
is a considerable body of evidence to support the contention
that this may be an important diagnostic parameter.
It seems unlikely that any single diagnostic analysis will
provide all the information required for the diagnosis and
management of urinary tract infections and a panel of tests
will almost certainly be required. To this end, experimental
models of urinary tract infection will continue to be useful in
the assessment of the potential of new and more established test
procedures.
FIGURE 95.

Four pathological conditions of the kidney including chronic pyelonephritis (CPN) defined on the basis of laboratory data (urinary antibody, histological examination of the kidney and the bacteriological examination of ureteric urine).
CHAPTER 17

RELATIONSHIP OF THE IMMUNOGENICITY OF THE INFECTING ORGANISM TO THE HOST RESPONSE IN URINARY TRACT INFECTION
INTRODUCTION

High titres of circulating antibody to the 0 somatic antigen of the homologous infecting E.coli strain are commonly found in patients with upper urinary tract infection. Frequently, however, even upper urinary tract infection fails to evoke an antibody response (Fairley, 1971). In this study, the immunogenicity of the infecting organism was investigated as a factor influencing the immune response of the host in pyelonephritis. Clinical aspects of the study were carried out by Drs. Fairley and Whitworth in Melbourne while the laboratory and experimental animal section of the protocol was performed in this laboratory.

MATERIALS AND METHODS

Subjects
Nine women and one man ranging in age from 22 to 66 years, all with recurrent renal infections confirmed by the Fairley bladder washout test (Fairley, 1971) were studied.

Vaccines
Two strengths were used, 50 x 10^6 and 1,000 x 10^6 organisms/ml. The organisms used were E.coli 06, 075 and 05 isolated from urinary tract infections associated with a high antibody response and E.coli 011 and a non-typable E.coli which had produced renal infection but no increase in circulating antibody titres.

The 06 and 011 vaccines were administered subcutaneously to patients in an initial dose of 0.1 ml. of 50 x 10^6 organisms/
ml. vaccine followed by doubling doses twice a week, to a maximum of $1,000 \times 10^6$ organisms. The $06$ vaccine was given to five women with upper urinary tract infection and persistently low antibody titres to the $0$ antigen of the homologous infecting organism. $011$ vaccine was given to four women and one man in whom a significant antibody titre had been demonstrated to an infecting organism of another coli serotype.

The vaccines were also tested experimentally in rats. Fifty adult rats (ten for each vaccine) were bled prior to vaccination for baseline titres and then injected intramuscularly with $0.2$ ml. of $1,000 \times 10^6$ organisms/ml. vaccine, and the vaccination repeated 10 and 20 days later. Blood samples for antibody titre were obtained 5, 10, 20 and 30 days after the initial challenge.

RESULTS

Results of vaccination in patients with recurrent urinary tract infection are shown in Table 26. Five patients had had documented upper urinary tract infection with persistently low antibodies to their infecting organisms. These patients received $06$ vaccine. Four showed a subsequent titre rise of at least two tube dilutions and titres of $1:640$. In the other patient, no such rise was demonstrated.

Five patients had high titres of antibody to the $0$ antigen of the homologous infecting organism. These patients received $011$ vaccine. The maximum titre obtained was only $1:160$.

Thus, following vaccination, a significant increase in serum antibody titre was found in four of five patients receiving $06$
TABLE 26.  (a) Haemagglutinating antibody response to 06 vaccine in "poor producers of antibody to 0 antigen expressed as the reciprocal of the titre.

(b) Haemagglutinating antibody response to 011 vaccine in "good antibody producers."
<table>
<thead>
<tr>
<th>Patient</th>
<th>Pre-treatment</th>
<th>1 month</th>
<th>2 months</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.Mc.C.</td>
<td>80</td>
<td>640</td>
<td>1280</td>
<td>640</td>
</tr>
<tr>
<td>P.V.</td>
<td>160</td>
<td>320</td>
<td>640</td>
<td>640</td>
</tr>
<tr>
<td>H.B.</td>
<td>80</td>
<td>640</td>
<td>320</td>
<td>320</td>
</tr>
<tr>
<td>E.A.</td>
<td>80</td>
<td>320</td>
<td>160</td>
<td>80</td>
</tr>
<tr>
<td>M.O.B.</td>
<td>20</td>
<td>40</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pre-treatment</th>
<th>1 month</th>
<th>2 months</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.McC.</td>
<td>40</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>S.S.</td>
<td>160</td>
<td>80</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>P.P.</td>
<td>40</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>A.J.</td>
<td>40</td>
<td>80</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>R.G.</td>
<td>20</td>
<td>160</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>
vaccine, in whom no such response had previously been demonstrable to their infecting organism, but no titre greater than 1:160 occurred after 011 vaccination in patients known to have produced antibody in high titre to a previous infection.

The results of vaccination of rats with the five E.coli strains are shown in Table 27. Peak titres were found five days after challenge and showed a slow decline over the 30-day interval. The three E.coli strains associated with high antibody titres in human urinary infection also proved to be highly immunogenic in the experimental animals. The response of the rat to challenge with the 011 and non-typable E.coli strains also paralleled the response in man and low antibody titres were found.

**DISCUSSION**

The results of challenging human subjects and rats with E.coli strains isolated from individuals who "responded" and failed to respond to renal infection with a significant rise in antibody titre has shown that the nature of the response may be governed by the immunogenicity of the infecting organism. When patients who had failed to respond to infection were challenged with a strain of E.coli isolated from a "responder" patient, four out of five individuals showed a significant increase in antibody levels to the challenging strain of E.coli. Similarly in a reciprocal study, patients who had responded to renal infection failed to respond to a challenge with the E.coli strain isolated from a "non-responder" patient.

The variable immunogenicity of the E.coli strains from the two
TABLE 27. Antibody response of individual rats following vaccination with 5 strains of E. coli isolated from patients with urinary tract infections.
<table>
<thead>
<tr>
<th>Rat No.</th>
<th>E. coli 05 Days after Challenge</th>
<th>Rat No.</th>
<th>E. coli 075 Days after Challenge</th>
<th>Rat No.</th>
<th>E. coli non-typable Days after Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>30</td>
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</tr>
<tr>
<td>1</td>
<td>0</td>
<td>1024</td>
<td>512</td>
<td>256</td>
<td>128</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>64</td>
<td>16</td>
<td>8</td>
<td>2</td>
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<tr>
<td>3</td>
<td>16</td>
<td>1024</td>
<td>512</td>
<td>512</td>
<td>256</td>
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<tr>
<td>4</td>
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<td>128</td>
<td>128</td>
<td>128</td>
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<tr>
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<td>8</td>
<td>256</td>
<td>64</td>
<td>64</td>
<td>64</td>
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<tr>
<td>6</td>
<td>8</td>
<td>256</td>
<td>64</td>
<td>64</td>
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<tr>
<td>7</td>
<td>8</td>
<td>64</td>
<td>64</td>
<td>32</td>
<td>32</td>
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<tr>
<td>8</td>
<td>0</td>
<td>128</td>
<td>128</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>512</td>
<td>512</td>
<td>256</td>
<td>128</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>64</td>
<td>32</td>
<td>64</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>E. coli 011 Days after Challenge</th>
<th>Rat No.</th>
<th>E. coli 06 Days after Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>16</td>
<td>DEAD</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>
groups of patients were confirmed in the animal experiments. Challenging rats with *E. coli* 06 and 05 responder strains resulted in high serum antibody titres whereas the 011 and non-typable strains did not induce any response in the challenged animals. The wide range in titres seen in the immunized rats (Table 27) and the poor response of patient M.O.B. (Table 26) also suggest that the genetically controlled ability of the host to respond to an immunogenic stimulus may also be a factor determining the extent of the immune response in individual cases.

These experiments provide an answer to the variable immune response of many patients to the homologous organism causing renal infection. No overall defect in the humoral immune capacity in this group has been found (Whitworth et al., 1972) and it appears that the immunogenicity of the infecting organism may be an important component determining the response of the host to infection.
CHAPTER 18

ANTIMICROBIAL RESISTANCE IN URINARY TRACT INFECTIONS
INTRODUCTION

Although most bacterial infections can now be controlled by antibiotic therapy, chronic urinary tract infection has proved to be an exception and prolonged freedom from infection is difficult to achieve. While there is agreement that treatment of urinary tract infections should be based on in-vitro sensitivity tests, there has been controversy over testing the sensitivity of pathogenic organisms at the concentration of antimicrobial found in the blood or encountered in the renal interstitium and urine.

In the clinical laboratory, the paper disc technique is the method commonly employed to determine the antibiotic sensitivity of isolates. With this method the diameter of the inhibition zone produced around a paper disc impregnated with the antibiotic is related to the antibiotic sensitivity of the organism. The test is calibrated to indicate the sensitivity of an organism at antibiotic levels attainable in the blood and tissue during normal therapy and cannot readily be adjusted to carry out sensitivity tests at antimicrobial levels encountered in the renal interstitium and urine.

Maximum urinary levels may reach a concentration over a hundred times greater than blood levels and sensitivity tests at these concentrations of antibiotic are commonly carried out using an agar-plate dilution procedure. This analysis is not carried out as a routine assay and there may be cases where pathogens reported as being resistant to a particular antibiotic by the disc technique, may in fact be sensitive to concentrations of
the antimicrobial encountered in the urine. In this investigation the antimicrobial sensitivity of a series of bacterial isolates from individuals with urinary tract infection was established. The tests were carried out by disc and agar-dilution methods to determine the extent to which organisms recorded as resistant by the disc method might be sensitive to concentrations of antibiotic encountered in the urine.

METHODS AND MATERIALS

Source of isolates
Laboratories from the major hospitals throughout New Zealand were asked to forward subcultures of Gram negative bacilli isolated from patients with significant bacteriuria and resistant to two or more antibiotics on routine sensitivity testing. One hundred and twenty isolates were received and identified by standard procedures for Gram negative bacilli.

Disc-plate sensitivity testing
The test is based on the fact that, for a given antibiotic, the size of the zone of inhibition is inversely related to the minimum inhibitory concentration. The method used has been described by Bauer et al., (1966).

Subcultures of the strains: On the day before testing, each strain was streaked onto a separate blood agar plate and incubated overnight at 37°C. to obtain individual colonies.

Preparation of inoculum: Several colonies were suspended in a tube containing 4 ml. of trypticase soy broth and incubated for four hours at 37°C. Suspensions were then prepared to give a density equivalent to that of a standard prepared by adding 0.5
ml. of 1% BaCl₂ to 99.5 ml. of 0.36 N H₂SO₄.

Disc sensitivity: 15 cm. glass Petri-dishes were filled to a depth of 6 mm. with Mueller-Hinton agar and the surface dried before inoculation. The bacterial suspension was spread on the medium surface using a cotton wool swab. After allowing a few minutes for the inoculum to dry, discs were placed on the agar and pressed lightly to ensure even contact. After overnight incubation at 37°C, the diameter of the zones of inhibition were measured. The disc potency and the interpretation of the zones of inhibition for the individual antibiotics used are given in Table 28 (Bauer et al., 1966).

Minimum inhibitory concentrations (MIC) determined by agar-plate dilution

Stock concentrations of the antibiotics being tested were diluted in Wellcotest agar (Burroughs Wellcome) to give a range of antibiotic concentration from 1000 µg./ml. to 7.7 µg./ml. Organisms for examination were subcultured overnight on blood agar and single colonies suspended in 5 ml. of sterile water. The bacterial suspensions were placed in the wells of a Clements antibiotic sensitivity replicator (H. Clements Pty. Ltd., Sydney, Australia) and seeded onto a 9 cm. Petri dish containing 35 ml. of agar. In this apparatus each replication loop delivered 5 µl. of fluid containing 2 x 10⁴ organisms over a 40 mm.² area. The plates were incubated overnight at 37°C and read next morning for inhibition of growth. All tests were carried out in duplicate.

Interpretation of the MIC determinations

The average concentration of antibiotic in the urine during
TABLE 28. Disc potency and zone size interpretation.
<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Disc potency</th>
<th>Resistant below</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>30</td>
<td>14</td>
</tr>
<tr>
<td>Nalidixic acid*</td>
<td>30</td>
<td>13</td>
</tr>
<tr>
<td>Furadantin*</td>
<td>300</td>
<td>14</td>
</tr>
<tr>
<td>Colymycin</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30</td>
<td>13</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30</td>
<td>13</td>
</tr>
<tr>
<td>Sulphafurazole</td>
<td>250</td>
<td>12</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10</td>
<td>13</td>
</tr>
</tbody>
</table>

* urinary tract infections only - relate to urine levels.
therapy was used to delineate sensitive from resistant organisms by the agar-plate-dilution method (Table 29). Organisms whose growth was inhibited by antimicrobial agents at concentrations below this figure were regarded as sensitive. The maximum concentration of antibiotic in the renal interstitium was obtained from the literature and wherever possible, has been based on the direct determination of the antimicrobial concentration in the renal lymphatics draining the interstitium. The source of this information has been collated in Table 29 and Figure 96.

RESULTS
Identification of Gram negative bacilli
The genus and number of the Gram negative bacilli are tabulated in Table 30. *Klebsiella* and *Escherichia* species were the most common organisms and accounted for 68% of the isolates examined. The remaining organisms were identified as belonging to the *Proteus* species, 17%, and *Enterobacter*, 9%. Six of the 120 isolates were from the *Pseudomonas*, *Alkaligines* and *Aeromonas* species.

Antimicrobial sensitivity
The results of determining the antimicrobial sensitivity of Gram negative bacilli from 120 individuals with significant urinary tract infections are presented in Figure 97 and the individual data summarized in Table 31. Each organism was tested against a panel of antibiotics and the diameter of the zone of inhibition determined by the disc-plate method and plotted against the MIC determined by agar-plate dilution. The
TABLE 29. Collation of reference material for the plasma, urine and interstitial concentrations of antimicrobial agents attained during therapy.
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Adult dose</th>
<th>Reference</th>
<th>Average urinary conc.</th>
<th>Reference</th>
<th>Maximum conc. in renal interstitium</th>
<th>Reference</th>
<th>Maximum conc. in plasma</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>400,000 units 6 hrly</td>
<td>(Stamey 1965)</td>
<td>75 µg./ml.</td>
<td>(Stamey 1965)</td>
<td>Same as plasma levels i.e. 9 µg./ml.</td>
<td>(Verwey 1966)</td>
<td>9 µg./ml.</td>
<td>(Miller 1948)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>500 mg. 8 hrly</td>
<td>(Brumfitt 1962)</td>
<td>250 µg./ml.</td>
<td>(Garrod 1963)</td>
<td>Twice plasma levels i.e. 12 µg./ml.</td>
<td>(Brown 1964)</td>
<td>6 µg./ml.</td>
<td>(Brumfitt 1962)</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>1 g. 6 hrly</td>
<td>(Garrod 1963)</td>
<td>300 µg./ml.</td>
<td>(Garrod 1963)</td>
<td>75% maximum plasma levels i.e. 14 µg./ml.</td>
<td>(Cockett 1967)</td>
<td>20 µg./ml.</td>
<td>(Tolhurst 1972)</td>
</tr>
<tr>
<td>Furadantin</td>
<td>100 mg. 6 hrly</td>
<td>(Stamey 1965)</td>
<td>100 µg./ml.</td>
<td>(Stamey 1965)</td>
<td>3 times plasma levels i.e. 7.5 µg./ml.</td>
<td>(Chisholm 1968)</td>
<td>2.5 µg./ml.</td>
<td>(Garrod 1973)</td>
</tr>
<tr>
<td>Colymycin</td>
<td>75 mg. 12 hrly</td>
<td>(Stamey 1965)</td>
<td>100 µg./ml.</td>
<td>(Froman 1970)</td>
<td>20 µg./ml.</td>
<td>(Stamey 1965)</td>
<td>6 µg./ml.</td>
<td>(Froman 1970)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>1 g. daily</td>
<td>(Garrod 1963)</td>
<td>300 µg./ml.</td>
<td>(Tisch 1958)</td>
<td>25 µg./ml.</td>
<td>author's estimate</td>
<td>25 µg./ml.</td>
<td>(Welch 1958)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>250 mg. 6 hrly</td>
<td>(Stamey 1965)</td>
<td>150 µg./ml.</td>
<td>(Stamey 1965)</td>
<td>3 times max. plasma levels i.e. 12 µg./ml.</td>
<td>(Cockett 1968)</td>
<td>4 µg./ml.</td>
<td>(Tolhurst 1972)</td>
</tr>
<tr>
<td>Sulphafurazole</td>
<td>2 g. then 1 g. 6 hrly</td>
<td>(Garrod 1963)</td>
<td>500 µg./ml.</td>
<td>(Garrod 1963)</td>
<td>Same as plasma level i.e. 100 µg./ml.</td>
<td>(Schlegel 1964)</td>
<td>100 µg./ml.</td>
<td>(Garrod 1973)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>80 mg. 8 hrly</td>
<td>(Garrod 1963)</td>
<td>50 µg./ml.</td>
<td>(Garrod 1963)</td>
<td>Same as plasma level i.e. 12 µg./ml.</td>
<td>(Chisholm 1968)</td>
<td>12 µg./ml.</td>
<td>(Gingell 1968)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>500 mg. 6 hrly</td>
<td>(Stamey 1969)</td>
<td>50 µg./ml.</td>
<td>(Stamey 1969)</td>
<td>75% of max. plasma levels i.e. 16 µg./ml.</td>
<td>(Cockett 1967)</td>
<td>25 µg./ml.</td>
<td>(Garrod 1973)</td>
</tr>
</tbody>
</table>
FIGURE 96.

A comparison of the plasma, urine and interstitial concentrations of individual antimicrobial agents attained during therapy.
TABLE 30. Genus and percentage of the Gram negative bacilli examined.
<table>
<thead>
<tr>
<th>Genus</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia</td>
<td>36</td>
<td>30</td>
</tr>
<tr>
<td>Klebsiella</td>
<td>45</td>
<td>38</td>
</tr>
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<td>Enterobacter</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Proteus</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Alkaligines</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Aeromonas</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
FIGURE 97.

Determination of antimicrobial sensitivity of Gram negative bacilli to individual antibiotics. Gram negative bacilli isolated from 120 individuals with urinary tract infections were tested (Following 10 plates).
Nalidixic acid

Minimum inhibitory conc. pg/ml

I I

Minimum inhibitory conc. μg/ml

Gentamicin

Minimum inhibitory conc. μg/ml
Minimum inhibitory conc. (µg/ml)

**Colistin**

**Nitrofurantoin**
Penicillin

Minimum inhibitory conc. µg/ml

Zone diameters in mm

Sensitive resistant

Tetracycline

Minimum inhibitory conc. µg/ml

Zone diameters in mm

Sensitive resistant

Minimum inhibitory conc. µg/ml

Average urine conc.

Interstitial conc.
Sulphafurazole

Kanamycin

Minimum inhibitory conc. μg/ml

Zone diameters in mm

Sensitive

Resistant

Average urine conc.

Interstitial conc.
of the organism have been given in Table 28. For 5 pages, the table gives details of the interpretation of the zone size in relation to the antibiotic sensitivity. Details of zone of inhibition determined by the disc method using the Kirby-Bauer method.

Interpreted into this agar diffusion using a replicate system and varying concentrations of the antibiotic

**MIC** = minimum inhibitory concentration of the antibiotic for the organism, indicates the concentration of the antibiotic.
<table>
<thead>
<tr>
<th>Mic</th>
<th>Plate</th>
<th>Mic</th>
<th>Plate</th>
<th>Mic</th>
<th>Plate</th>
<th>Mic</th>
<th>Plate</th>
<th>Mic</th>
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<td>3.08</td>
<td>0</td>
<td>3.08</td>
<td>0</td>
<td>3.08</td>
<td>0</td>
<td>3.08</td>
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<tr>
<td>0</td>
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<td>1.5</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>1.5</td>
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<td>TABLE 32. Antibiotic sensitivity of Gram negative bacilli from individuals with urinary tract infection. Isolates were tested for antibiotic sensitivity by a disc procedure and the minimum inhibitory concentration determined by agar-plate dilution.</td>
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<td></td>
<td>Tetracycline</td>
<td>Fucidacin</td>
<td>Cephalothin</td>
<td>Kanamycin</td>
<td>Neomycin</td>
<td>Nalidixic acid</td>
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<td>Colymycin</td>
<td>Sulphafurazole</td>
<td>Gentamicin</td>
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<tr>
<td>R&lt;sup&gt;st&lt;/sup&gt; disc method</td>
<td>95</td>
<td>54</td>
<td>50</td>
<td>34</td>
<td>107</td>
<td>37</td>
<td>120</td>
<td>23</td>
<td>85</td>
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<td>R&lt;sup&gt;st&lt;/sup&gt; disc and R&lt;sup&gt;st&lt;/sup&gt; agar diln. at ur. levels</td>
<td>27</td>
<td>20</td>
<td>13</td>
<td>13</td>
<td>89</td>
<td>33</td>
<td>109</td>
<td>20</td>
<td>83</td>
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<td>R&lt;sup&gt;st&lt;/sup&gt; disc but sens. agar diln. at ur. levels</td>
<td>68</td>
<td>34</td>
<td>37</td>
<td>21</td>
<td>18</td>
<td>4</td>
<td>11</td>
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<td>(72%)</td>
<td>(63%)</td>
<td>(74%)</td>
<td>(62%)</td>
<td>(17%)</td>
<td>(11%)</td>
<td>(9%)</td>
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<td>R&lt;sup&gt;st&lt;/sup&gt; disc. but sens. agar diln. at interstit. levels</td>
<td>1</td>
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<td>2</td>
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<td>1</td>
<td>10</td>
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zone sites below which the organisms were considered resistant are indicated on the individual figures together with the average urinary concentration of antimicrobial agent and the maximum interstitial level.

As expected, many of the results from these analyses fell into either the two upper right segments of each figure, i.e. sensitive by the disc method and at urinary concentration of antibiotic, or the lower left segment, i.e. resistant by disc analysis and also resistant at concentrations of antibiotic attainable in the urine. A significant number of the organisms was found to lie in the central lower segment indicating resistance to the antimicrobial agent by disc analysis but sensitivity at levels of antibiotic attainable in the urine.

Seventy-two % of the organisms resistant to cephalothin by the disc method were sensitive to average urinary levels of antimicrobial agent (Table 32). Similar results were obtained for gentamicin 75%, although only four resistant organisms were found, tetracycline 72%, furadantin 63%, kanamycin 62%, ampicillin 17%, nalidixic acid 11% and penicillin 9%. The majority of organisms resistant by disc analysis to sulphafurazole were found to be resistant also at urinary concentrations. In the case of penicillin, isolates sensitive at interstitial and urinary levels were all found to belong to the Proteus genus and represented 55% of the Proteus strains examined.

DISCUSSION

The results of testing the antimicrobial sensitivity of 120 isolates from individuals with renal infection at blood,
urinary and renal interstitial levels of antimicrobial agent have shown that up to 75% of the organisms resistant by standard disc procedures may be sensitive at concentrations of antibiotic readily attainable in the urine.

In recent years, factors governing the treatment of urinary tract infections have largely been determined by the prevalent interpretation of the relationship between proven infection and pathological changes. Concepts of the pathogenesis of chronic pyelonephritis have changed considerably over the last few years and the role of bacterial infection per se as a major cause of chronic pyelonephritis has been questioned (Freeman, 1973).

Other developments have also influenced the therapy of urinary tract infection. Procedures for the localization of infection (Fairley et al., 1971; Thomas, Shelekov & Forland, 1974; Jones, Smith & Sanford, 1974) and studies in general practice (Gallagher, Montgomerie & North, 1965) have determined the association between symptoms and actual infections and have provided a basis for identifying individuals that require a more aggressive therapeutic approach.

Arguments have been raised that elimination of bacteria from the urine does not necessarily indicate elimination of bacteria from the renal parenchyma and that these two situations should be considered in isolation. This seems unwarranted and until further studies have established the relationship between infection and pathological changes, and the contribution of continuing bacteriuria to progressive kidney damage, it is reasonable to accept Stamey's argument that the urine bacteriology...
reflects the status quo in the renal parenchyma as well as in the lower urinary tract (Stamey, Govan & Palmer, 1965). At the very least, sterilization of the urine removes a focus for continuing infection which is clearly of importance where infection is associated with obstruction, diabetes mellitus, analgesic nephropathy or infection during pregnancy.

The foregoing factors have been considered in initiating the current experiments. A dominant point of view has been that successful treatment of renal infection can only be achieved by maintaining an effective antibiotic concentration at the site of infection. This has led to the practice of testing for antibiotic sensitivity at attainable blood levels as the cortex is particularly vascular and therapeutic levels of the antibiotic should be readily attained in the tissues. This view has been accepted with some reservations as it is also believed that the high concentration of antibiotic in the urine may lead to a greater concentration of antibiotic in the medullary tissue than in the cortex.

Because of the technical difficulties of separating interstitial fluid from the urine contained in the tubular lumen of the medulla, it has not been possible to determine the concentration of antibiotic in the medulla or the cortical interstitium. Recently, however, cannulation of the lymphatics draining the interstitium has allowed a more direct measurement of the concentration of antibiotic in the interstitium. From these studies it is clear that the concentration of antibiotic in the interstitium never approaches urinary levels although in some cases the interstitial concentrations may be several fold
higher than serum levels. Even the high concentrations obtained during anti-diuresis had no apparent effect on the concentration of antibiotic in the renal lymph. Experiments with gentamicin illustrate this point as recent studies have shown that antibiotic levels in lymph from the thoracic duct and kidney were entirely unaffected by high urine levels (Chisholm, Calnan & Waterworth, 1968). Nitrofurantoin, however, may be an exception as the drug is secreted into the proximal tubules and then reabsorbed from the distal tubule (Buzard et al., 1962; Woodruff, Malvin & Thompson, 1961).

The case for antibiotic sensitivity testing at higher concentrations of antibiotic assumes that pathogens resistant at blood levels may be sensitive at the higher antibiotic levels attainable in the urine. The assumption is not unreasonable as most organisms acquire antibiotic resistance as a stepwise procedure and the extent of resistance is usually not absolute but a question of degree. The present results have confirmed this and up to 75% of the isolates resistant by standard disc procedures were found to be sensitive to urinary levels of antibiotics. A high proportion of the Proteus group were resistant at blood levels of penicillin but sensitive to interstitial levels of the antibiotic. There seems to be little doubt, however, that the importance of testing for antibiotic sensitivity at interstitial levels has been over-emphasized.

One final point deserves special attention. If the antibiotic concentration in urine can reach potentially effective levels, would the restriction of fluids to increase the level of antibiotic still further seem advisable. Arguments for both
the forcing of fluids and the restriction of fluids in pyelonephritis have been presented and supported by the results from experimental infection (Andriole & Epstein, 1965; Miller & North, 1966; Kaye, 1968). Even the experimental results have not been conclusive, however, and some reports have suggested a favourable response to diuresis where others have found the reverse effect (D'Alessio et al., 1971; Freedman, 1967; Kaye, 1971).

This dispute seems to have been clarified by the clinical and bacteriological observations of Cattell et al., (1968) in a study of the effects of diuresis and antibiotic therapy in urinary tract infection. An increased urine flow diluting the concentration of antimicrobial would seem to be a disadvantage during antibiotic therapy but surprisingly the reverse was found. Previous reports by the same group had indicated that any effect of antibiotic dilution was more than compensated for by the combination of diuresis and antibiotic therapy (O'Grady & Cattell, 1966) and this was shown to be the case in the clinical experiments. A significant reduction in the bacterial count of bladder urine was found in patients being treated with a high fluid intake combined with antibiotic therapy despite a reduction in the concentration of antibiotic in the urine.

These conclusions have been confirmed by recent experimental studies where enterococcal pyelonephritis was treated with a variety of regimens. Under the conditions of the experiment, furosemide-induced diuresis, together with ampicillin treatment reduced the numbers of bacteria in the kidney significantly more than ampicillin or diuresis alone (Levison & Kaye, 1973).
Taken overall, these experiments justify determining the minimum inhibitory concentration of selected antimicrobial agents in cases of recalcitrant urinary tract infection caused by Gram negative bacilli. The procedure may indicate potentially effective antibiotics despite a resistance report by routine analysis and extend the range of antibiotics available to the physician.
Induction of experimental pyelonephritis in the rat by the direct inoculation of E. coli into the kidney (Chapter 2).

In these experiments, a consistent and reproducible infection in the rat was produced using a micropipette to introduce bacteria directly into the kidney. The gross appearances and histopathologic changes were similar to those seen during the course of the disease in man. Bacteria were found to persist for extended periods in the kidney but in contrast to other reports, persistent infection was not confined to the medullary region. The model appears to have advantages over models currently available and permits the establishment of a pyelonephritic process in prescribed areas of renal parenchyma.

Experimental pyelonephritis: the specificity of the inflammatory lesion (Chapter 3).

The induction of experimental renal infection produces damage to the kidney parenchyma so that the histopathological changes do not necessarily represent the response of the host to infection alone. The degree of renal damage, and the nature of the cellular infiltrate in experimental renal infection and a variety of other types of sterile injury were compared to differentiate the non-specific element from specific features of the pyelonephritic lesion. It was possible to demonstrate that in addition to an inflammatory and repair process which is common to tissue injury, there were also quantitative and qualitative changes in the cellular infiltrate specifically associated with active infection. The degree of the lymphocytic
infiltrate, the presence of large numbers of plasma cells and foci of PAS-staining histiocytes were specific features associated only with pyelonephritis.

Glomerular filtration rate in the rat using $^{51}$Cr-EDTA administration and a single blood sample (Chapter 4). $^{51}$Cr-EDTA was used to measure the glomerular filtration rate in rats using external body counting and a two compartmental analysis according to the method of Sapirstein. A comparison was made of results calculated using the two compartment method and a formula that requires a knowledge of the distribution volume of the isotope and the analysis of a single timed plasma sample. The results have shown that the formula of Bryan et al. can be used for the rapid and convenient determination of glomerular filtration rate in small animals following the administration of $^{51}$Cr-EDTA.

Experimental pyelonephritis: chronic active pyelonephritis and renal function (Chapter 5). Renal function in chronic active pyelonephritis was investigated and the effect of antibiotic treatment and elimination of infection on the gross pathology, histopathology and renal function in animals with chronic pyelonephritis determined. A severe loss of urine concentrating capacity was demonstrable when the maximum urinary osmolality of a group of animals with pyelonephritis were compared with control animals. Concentrating capacity decreased sharply over the first month, but further loss over an 8-month period was minimal. A compensatory increase in the glomerular filtration rate (GFR) in the control, non-
challenged group occurred after nephrectomy but no comparable compensation in the infected group was found. Antibiotic therapy had a marked effect on the urinary concentrating capacity and the defect in concentrating ability was significantly less in the treated animals during the first 30 days after challenge. Infection again prevented a compensatory increase in the GFR of pyelonephritic animals, but this was not reversed by antibiotic therapy. Blood urea levels in treated and non-treated animals were not significantly different nor did the eradication of infection affect the gross pathology and histopathological changes found at autopsy.

Experimental pyelonephritis: establishment of retrograde renal infection (Chapter 6).

Current experimental models for the induction of retrograde renal infection have several limitations and it is usually necessary to damage the kidney to establish infection. In these experiments a method for producing infection in the unmanipulated kidney was developed. A reservoir of the bacterial inoculum was retained in a small glass capsule and placed in the bladder. The slow release of the inoculum into the bladder lumen led to lower urinary tract infection and ultimately the establishment of renal infection in 85% of the animals challenged.

Bacterial interference as a determinant in the epidemiology of urinary tract infection (Chapter 7).

These experiments have investigated the role of bacterial interference as a determinant in the epidemiology of renal
infection. Two unrelated strains of *Escherichia coli*, E.coli 08 and 075, isolated from cases of clinical pyelonephritis were used. Although both strains had identical morphology on conventional media, they could be differentiated using genetically stable markers for streptomycin resistance and arabinose utilisation. When the two strains of E.coli were introduced into the kidney simultaneously by direct inoculation, mixed infections were readily established. On the other hand, although both strains of E.coli were equally invasive as individual pathogens, pyelonephritis, when induced using a retrograde challenge with a mixed culture of the same organisms was almost invariably caused by the 08 strain alone. Further experiments showed that bacterial interference occurred within the kidney and determined the pattern of infection. When unilateral renal infections were established with E.coli 08 and the animals subsequently challenged with E.coli 075, it was found that E.coli 075 infection never occurred in kidneys infected with E.coli 08, but infection was established in the contralateral kidney. The experiments have shown that mixed renal infections with E.coli are uncommon even when both pathogens are equally nephropathogenic and are introduced simultaneously into the bladder.

Acquiescent infection as a factor in pyelonephritis (Chapter 8). The relationship between bacterial infection of the renal parenchyma with *Escherichia coli* and the establishment of pathological lesions was investigated experimentally. Infection was established in one kidney and the bacteriological, pathological and immunological features of infection were compared in the
pyelonephritic and contralateral unmanipulated kidney. Whereas active bacterial infection was associated with pathological changes in the pyelonephritic kidney, a poor correlation was found between bacterial growth and the gross pathology and histopathological changes in the contralateral kidney. The conclusion from these studies was that infection of the kidney is not always associated with pathological changes. The term "acquiescent infection" has been used to describe this host-parasite relationship in which active, persistent, bacterial infection is not associated with pathological lesions. Evidence was presented that bacteria in the contralateral unmanipulated kidney are present in the renal parenchyma and that bacterial proliferation can be induced following renal trauma. Activation of infection and bacterial proliferation did not always result in pathological changes to the kidney and was not associated with an increase in serum antibody.

The in vitro response of rat lymphocytes to mitogens (Chapter 9). A simple but dependable method for determining the response of rat lymphocytes to mitogens in culture was described. The in vitro culture conditions for determining the incorporation of tritiated thymidine after stimulation with phytohaemagglutinin and other mitogens were given in detail, and the methodology confirmed by measuring the ablation of T cell responsiveness to PHA in T lymphocyte depleted animals.

Antigen presentation as a factor in the protective immune response to renal infection (Chapter 10). Host protection against renal infection may be augmented by
active immunization against the causative organism. In these experiments the effect of varying amounts and methods of presentation of bacterial antigen on the secondary immune response was investigated. Primary immunization with varying amounts of both killed and live antigen did not affect the nature of the secondary immune response although active renal infection did have a noticeable effect on the titre of serum antibody during the primary immune response. The experiments confirmed the presence of immunological memory to the somatic antigen of *E. coli* and showed that memory persisted for at least six months after primary immunization. Experiments have also been carried out which have demonstrated that memory to the somatic antigen of *E. coli* is carried by the B lymphocyte.

**Quantitation of immunoglobulin-bearing lymphocytes and the lymphocyte response to PHA in experimental pyelonephritis** *(Chapter 11).*

In these experiments, the effect of experimental pyelonephritis on the distribution of B lymphocytes in the peripheral blood and lymphoid sites in the rat was determined and the functional capacity of T cells during the course of infection investigated. The studies have shown that renal infection affects the distribution of lymphocytes and has a marked effect on the functional capacity of splenic T lymphocytes early in infection. Most of the lymphocytes forming the round cell infiltrate in the kidney were identified as thymus derived lymphocytes by their surface labelling characteristics. Evidence was presented to demonstrate the inability of T lymphocytes to function normally in the environment of the kidney, suggesting that ablation of
cell-mediated immunity may be a factor contributing to the persistence of infection in pyelonephritis.

The nature of the factor associated with renal cells causing ablation of T lymphocyte function (Chapter 12). In the previous series of experiments it was shown that the PHA responsiveness of normal lymphocytes in vitro could be blocked by the addition of normal kidney cells to the culture. Experiments were then carried out to characterize the factor causing ablation of the PHA response. The experiments showed that renal cells added to PHA stimulated splenic lymphocytes depressed the PHA response when as few as 2% of the cells in culture were renal cells. When a suspension of lung cells was added to lymphocyte cultures in the same way, ablation was not found. Cell to cell contact was not necessary, nor was renal cell viability essential and the ablation factor could be prepared from disrupted renal cells. Two active components were demonstrated; one, a small molecular weight product was capable of passing through a dialysis membrane in a double chamber culture vessel and the other, a substance of higher molecular weight, was non-dialysable but highly active. Neither substance was toxic for rat lymphocytes. On the basis of its heat stability, its specificity for rat but not human lymphocytes the ablation factor was not thought to be a lymphocyte chalone and seemed to have unique properties.

Selective deficiency of thymus derived lymphocytes in experimental pyelonephritis (Chapter 13). Pyelonephritis was induced in experimental animals deprived of
thymus derived (T) lymphocytes by adult thymectomy and serial sublethal irradiation. In this model, T lymphocytes were reduced to less than 1% of normal adjudged by the in vitro PHA responsiveness of lymphoid cells from deprived animals compared with control animals. Pathological, bacteriological and immunological aspects of renal infection were studied in the T cell deprived animals during the acute, resolving and chronic stages of pyelonephritis. The experiments have shown that the ablation of T lymphocytes did not appreciably alter the course of the disease.

Immunological enhancement in the pathogenesis of renal infection (Chapter 14).

The role of the immune response in renal infection was investigated by manipulation of the host's immune capacity using the immunosuppressive drugs, 6-mercaptopurine, cyclophosphamide and thiamphenicol. Treatment with 6-mercaptopurine depressed the humoral immune response but did not have an adverse effect on the course of renal infection. Thiamphenicol administration prevented the development of pathological lesions but this was due to the antibacterial activity of thiamphenicol and not its immunosuppressive activity. Pyelonephritic animals treated with cyclophosphamide did not produce antibacterial antibody. Despite this, cyclophosphamide treated animals were able to eliminate organisms more readily from the infected kidney than untreated animals with a normal humoral immune response. This suggests that blocking of the phenomenon of immunologic enhancement may explain these unexpected results and that the immune response to renal infection could have an immuno-
enhancing role, protecting the bacterial cell from otherwise effective host defense mechanisms.

Determination of serum antibody levels during the immune response to bacterial antigen using a haemagglutinating autoanalyser (Chapter 15). Currently there are no convenient procedures available for the estimation of antibacterial antibody on a continuous scale and antibacterial titres are usually reported as the reciprocal of the highest dilution of the sample giving agglutination. In this chapter, the adaption of a haemagglutinating autoanalyser for the determination of antibacterial antibody was described. Titres were reported on a continuous scale that was not restricted to the limitations of serial dilution end points. The method was found to be reproducible, specific and show increased sensitivity when compared with manual methods.

Diagnostic parameters in experimental renal infection (Chapter 16).

Urinary tract infections are commonly encountered in medical practice but their laboratory diagnosis presents many difficulties. In these experiments, models of the disease were used to assess the value of serum antibody, urinary antibody, rheumatoid factor, and the histo-chemical examination of renal tissue as diagnostic parameters. Under the conditions of the experiment, the analyses did provide useful diagnostic information and may prove to be of value in the management of urinary tract infection in man.
Relationship of the immunogenicity of the infecting organism to the host response in urinary tract infection (Chapter 17).

In this study, the immunogenicity of the infecting organism was investigated as a factor influencing the immune response of the host in pyelonephritis. Experiments in both man and animals showed that the humoral immune response to renal infection is largely determined by the immunogenicity of the causative organism.

Antimicrobial resistance in urinary tract infections (Chapter 18).

In this investigation, Gram negative bacilli resistant to two or more antibiotics were isolated from 120 individuals with urinary tract infection. Antibiotic sensitivity patterns were determined by the Kirby-Bauer paper disc method and by an agar-plate-dilution method capable of measuring the minimum inhibitory concentration at the level of antibiotic attainable in the renal interstitium and urine. The results showed that in the case of selected antibiotics up to 75% of the isolates resistant by standard disc procedures were sensitive at concentrations of antibiotic readily attainable in the urine. The implications of these results in relation to the therapy of urinary tract infection were discussed and evidence presented that the determination of the minimum inhibitory concentration of selected antibiotics may be of considerable value in the management of urinary tract infections.
ACKNOWLEDGEMENTS

This study was carried out during my tenure of the Isaacs Memorial Medical Research Fellowship and I am deeply indebted to the Trustees for their financial support. Financial assistance by the New Zealand Medical Research Council, who provided funds for essential equipment and material is also gratefully acknowledged. Professor J.D.K. North kindly provided facilities in the Department of Medicine and his interest, advice and support was appreciated. Many of the experiments for investigating the immunobiology of pyelonephritis arose out of discussions with Dr. J.D. Wilson and it is a pleasure to acknowledge his guidance and critical assessment which contributed to all aspects of this investigation. A number of experiments involved irregular hours, weekend and holiday commitments and I am very grateful to the laboratory staff in the Department of Medicine for their support in maintaining demanding experimental protocols over these periods. Mrs. Sue Phillips was closely associated with me during the course of this work and her attention to the collection and storage of samples, collation of data and assistance with the compilation of the literature related to this thesis is acknowledged with gratitude. Mr. Roy Douglas kindly made the autoanalyser in the Blood Transfusion Centre available and provided expert advice during the development of a method for the automated determination of antibacterial antibody. My wife, Helen, assumed many additional family responsibilities without complaint and her unfailing support made this study possible. The skilled and critical secretarial assistance of Mrs. Eve Smith who worked long hours to present a flawless thesis is also acknowledged. She eliminated many errors; the ones remaining are mine.
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