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Selective recruitment of lymphocyte subsets to the inflamed appendix

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SUMMARY

Total lymphocyte counts and the distribution of lymphocyte subsets were determined in peripheral venous blood and appendiceal mononuclear cells from 60 patients who underwent appendicectomy for the clinical diagnosis of appendicitis. A significant peripheral lymphopenia was observed in the 46 patients with histologically confirmed acute appendicitis which was accompanied by an increase in the appendiceal lymphocyte concentration. There was an even greater depletion of $CD45RO^+$ (memory) T lymphocytes in peripheral blood and an increase in the inflamed appendix. Reciprocal changes were observed in the $CD45RA^+$ (naive) T lymphocyte subset. These changes were reflected in the local arterial and venous CD45RA and CD45RO T lymphocyte subsets. Proliferation studies showed an expanded functional repertoire of T lymphocytes in the inflamed appendix. Selective recruitment of memory T lymphocytes from the peripheral blood to the inflamed appendix was demonstrated.

Keywords appendicitis lymphopenia CD45 naive lymphocytes memory lymphocytes

INTRODUCTION

Peripheral blood lymphopenia often accompanies sepsis. Lymphopenia has been shown to occur in acute appendicitis [1], acute diverticulitis [2] and acute pancreatitis [3]. We were interested to know the fate of lymphocytes which left the peripheral blood. Did they migrate to sites of inflammation? And was migration confined to certain subsets of lymphocytes?

Acute appendicitis provides an unique opportunity to investigate these questions. It is common; approximately 250 appendicectomies are performed every year in our hospital. It occurs in all age groups and has no gender differences. It is the only common acute inflammatory condition in which the inflamed organ is completely removed and available for analysis. Equally importantly, there are false positive diagnoses, making apparently normal appendices available for comparison. This clinical situation offers an opportunity to investigate lymphocyte traffic in acute inflammation.

PATIENTS AND METHODS

Patients

Sixty patients with the clinical diagnosis of acute appendicitis were studied. The mean age was 24·7 years, range 16–73 years. There were 27 males and 33 females. Patients under the age of 16 were excluded due to technical and ethical considerations. Out of the 60 patients, 46 had histologically confirmed acute appendicitis, one had an urinary tract infection, while the remaining 13 had no demonstrable pathology and were classified as having non-specific abdominal pain. The project was approved by the Whittington Hospital ethics committee.

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Lymphocyte sampling and preparation

A 13-ml pre-operative venous blood sample was collected from each patient, of which 3 ml were placed in an EDTA-coated glass tube (Becton Dickinson, Oxford, UK) for automated full blood count on a Technicon H-1 automatic analyser. The residual 10 ml were placed in a heparin-coated endotoxin-free glass tube (Becton Dickinson). The heparinized blood samples were later diluted with two volumes of PBS.

The distal 0·25 cm at the tip and the proximal 0·25 cm at the base of the appendices were removed for routine histology. The rest of the appendices were laid open and left to stand for 2 h at room temperature in culture medium (RPMI 1640) containing 5% complement-depleted fetal calf serum (FCS), 4 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were removed from the appendices by blunt dissection in sterile Petri dishes containing culture medium. Histological examination of residual tissue after this procedure confirmed the efficient and complete harvesting of mononuclear cells from the appendices. The volumes of appendices were measured by fluid displacement before and after cell collection. Cell counts were carried out in a haemocytometer.

The diluted blood and the appendiceal cells were layered onto Ficoll–Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 250 g for 25 min at 4°C for density separation.

The interphase layer containing mononuclear cells was removed and washed twice in PBS.

Local appendiceal arterial and venous samples were separately collected at the time of operation from five patients with appendicitis and five patients with histologically normal appendices. The main appendiceal vein was dissected out and divided. Approximately 1 ml of local venous blood was collected into a sterile Eppendorf tube containing 250 μl PBS with 1 U of heparin. The main appendiceal artery was then severed and local arterial blood collected into another similar Eppendorf tube. Ficoll–Paque density separations were carried out.

Lymphocyte labelling

Nine MoAbs were used for the phenotyping: anti-CD3 (UCHT1) [4] for all T lymphocytes, anti-CD4 (QS4120) [5] for helper T lymphocytes, anti-CD8 (UCHT4) [6] for cytotoxic T lymphocytes, anti-CD19 (BU12) [7] for B lymphocytes, anti-CD25 (Tac) [8] for IL-2 receptors (IL-2R), anti-CD45RA (SN130) [9] for naive cells, anti-CD45RO (UCHL1) [10] for memory cells, WT31 [11] for $\alpha\beta$ T cells and TCR-γ/δ1 [12] (T-Cell Sciences, Cambridge, MA) for $\gamma\delta$ T cells.

Single labelling was carried out with anti-CD19 and anti-CD25 antibodies. Cells were stained in round-bottomed polyvinyl microtitre plates (Falcon, Oxford, UK). One hundred thousand cells per well were suspended in 100 μl PBS and a saturating amount of the primary MoAb was added. They were incubated for 30 min at 4°C. The cells were then washed twice in PBS with 1% FCS which was used in all subsequent washes. They were then incubated with a saturating concentration of FITC-conjugated goat anti-mouse immunoglobulin $F(ab')_2$ fragment (Sigma, Poole, UK) for 30 min and washed twice.

Double staining was carried out using a four-step method. This was to establish the proportions of various subsets within the T lymphocyte population. One hundred thousand cells per well were suspended in 100 μl PBS. They were incubated with a saturating amount of monoclonal anti-CD4, anti-CD8, anti-CD45RA, anti-CD45RO, anti-$\alpha\beta$ or anti-$\gamma\delta$ antibody for 30 min at 4°C and washed twice. The cells were then incubated with a saturating concentration of FITC-conjugated goat anti-mouse immunoglobulin $F(ab')_2$ fragment for 30 min at 4°C and washed twice. They were resuspended in 100 μl PBS with 1:25 normal mouse serum (Dako, High Wycombe, UK) for 15 min to block non-specific binding, and were washed twice again. The final incubations were carried out with a saturating amount of PE-conjugated anti-CD3 MoAb for 30 min at 4°C, after which the cells were again washed twice.

Acquisitions and analyses were carried out with a Becton Dickinson FACScan flow cytometer. The lymphocyte population was defined by its forward and side scatter characteristics. Ten thousand events were acquired for each sample. The FITC label was detected by the FL1 channel, while the PE label was detected by the FL2 channel. A FITC control was performed by incubating a sample with only the FITC second layer antibody, and a PE control by adding only PE to sample. Controls were washed twice before analysis. Background binding was deducted from experimental results. The percentages of B lymphocytes ($CD19^+$), T lymphocytes ($CD3^+$) and activated cells ($CD25^+$) were calculated in relation to a total lymphocyte gate using fluorescence histograms. Quadrant plots of FL1 *versus* FL2 were used to determine the numbers of CD4, CD8, CD45RA, CD45RO, $\alpha\beta$ or $\gamma\delta$ positive cells as percentages of $CD3^+$ cells.

Proliferative responses of T lymphocytes

Peripheral blood and appendiceal mononuclear cells from six patients with appendicitis and six patients with normal appendices were tested for their proliferative response to various stimuli. The cells were resuspended at a final concentration of $2{\cdot}5 \times 10^6$ cells/ml in RPMI 1640 with 10% complement-depleted pooled human AB serum (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin and 4 mM glutamine. Quadruplicate 200-μl samples were plated in flat-bottomed microtitre plates (Falcon). The following stimuli were used: phytohaemagglutinin (PHA; Wellcome, Dartford, UK) at 1 μg/ml, influenza antigen (Flu) (X31, National Institute for Medical Research, London, UK) at 200 haemagglutination units (HAU)/ml, tuberculin purified protein derivative (PPD; Evans, Leatherhead, UK) at 100 U/ml, tetanus toxoid (TT; Wellcome) at 2 U/ml, a mitogenic pair of anti-CD2 MoAbs (OKT11 and GT2) [13,14] at 1:20 dilution of culture supernatant and anti-CD3 culture supernatant (UCHT1) [4] at 1:20 dilution. The optimal final concentration of each stimulus had previously been determined. The cells were incubated at 37°C and 5% carbon dioxide. The PHA, anti-CD2 and anti-CD3 stimulated cells were incubated for 48 h. The Flu, TT and PPD-stimulated cells were incubated for 120 h. Separate controls were carried out for the two groups. At the end of the incubation, 0·5 μCi of ^{3}H-methyl-thymidine (Amersham, Aylesbury, UK) was added to each well. The wells were harvested onto glass fibre filters 6 h later. T lymphocyte proliferation is proportional to ^{3}H-methyl-thymidine uptake, which was assessed by liquid β-scintillation counting in an automated reader (LKB, Milton Keynes, UK). The ct/min was averaged for the quadruplicates.

Statistical analysis

Geometric means were used throughout, as the data were not normally distributed. Statistical analyses were carried out where indicated.

RESULTS

Lymphocyte counts

Peripheral lymphopenia was observed in patients with histologically inflamed appendices. The mean peripheral blood lymphocyte count was $1{\cdot}89 \times 10^9/l$ in patients with normal histology and $1{\cdot}12 \times 10^9/l$ in patients with acute appendicitis ($P < 0{\cdot}05$, Mann–Whitney two-tailed test). We also observed increases in lymphocyte counts in the appendices when they were inflamed. The appendiceal mean lymphocyte count was $2{\cdot}1 \times 10^9/l$ in patients with normal histology and $5{\cdot}8 \times 10^9/l$ in patients with acute appendicitis ($P < 0{\cdot}05$, Mann–Whitney two-tailed test). These findings suggested an accumulation of lymphocytes in the inflamed appendices. A negative correlation existed between the peripheral lymphocyte count and the appendiceal lymphocyte count (Spearman's correlation $r = -0{\cdot}74$, $P < 0{\cdot}05$). Therefore some lymphocytes in the inflamed appendices might have come from the peripheral blood.

Peripheral blood and appendiceal lymphocyte subsets

In appendicitis, there was a significant reduction in peripheral

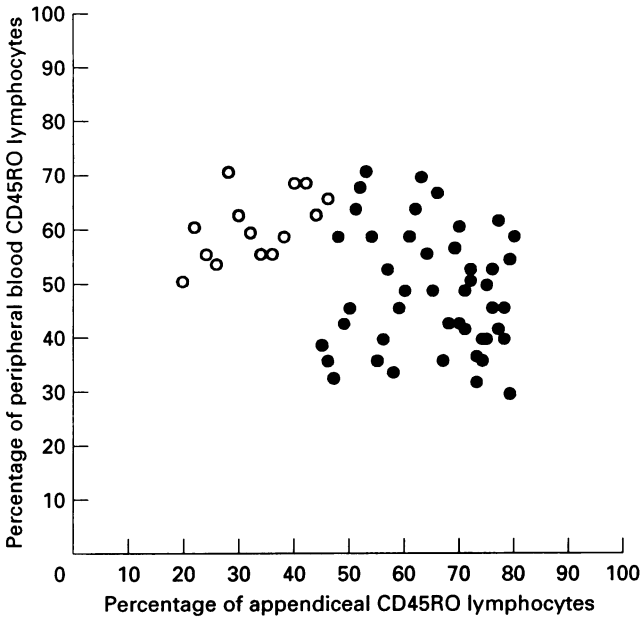

Fig. 1. Memory T lymphocytes ($CD45RO^+$) as percentages of total T lymphocytes ($CD3^+$) in the appendix and peripheral blood. ○, Patients with normal appendices; ●, patients with appendicitis.

blood memory T lymphocytes (CD45RO and CD3 double-positives) as a percentage of total T lymphocytes (CD3-positives) ($P < 0{\cdot}001$, Mann–Whitney two-tailed test) (Fig. 1, Table 1). The percentage of memory T lymphocytes increased in the inflamed appendices ($P < 0{\cdot}001$, Mann–Whitney two-tailed test) (Fig. 1, Table 1). Reciprocal changes were observed in the naive T lymphocyte population. There was an increase in peripheral blood naive T lymphocytes (CD45RA and CD3 double-positives) as a percentage of total T lymphocytes in

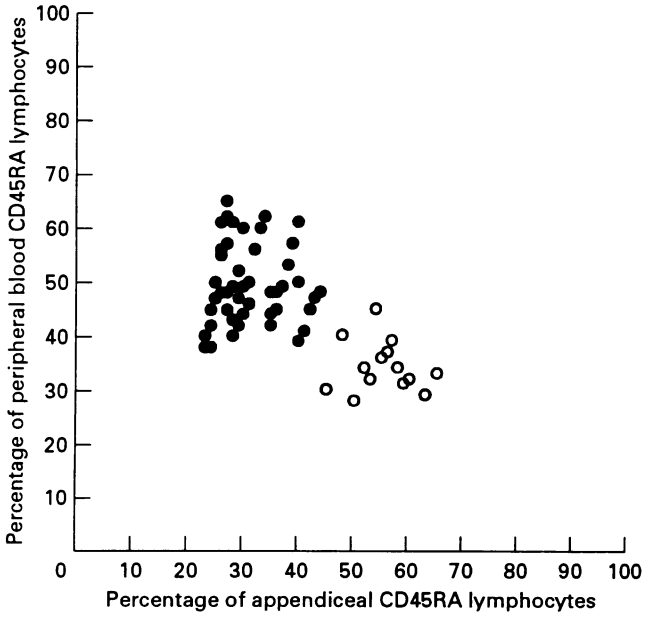

Fig. 2. Naive T lymphocytes ($CD45RA^+$) as percentages of total T lymphocytes ($CD3^+$) in the appendix and peripheral blood. ○, Patients with normal appendices; ●, patients with appendicitis.

Table 1. Mean percentages (range) of various lymphocyte subsets in blood and appendix of 46 patients with appendicitis and 14 patients with histologically normal appendices

	Normal histology		Appendicitis	
	Blood	Appendix	Blood	Appendix
$CD4^+$ T cells	62 (55–67)	45 (39–48)	59 (52–68)	41 (36–49)
$CD8^+$ T cells	41 (38–46)	63 (54–69)	43 (37–49)	66 (59–71)
$CD45RO^+$ T cells	60 (48–69)	33 (22–49)	48 (29–71)	65 (47–80)
$CD45RA^+$ T cells	34 (28–45)	55 (47–68)	49 (37–65)	31 (26–58)
$\alpha\beta$ T cells	94 (88–98)	91 (82–96)	95 (88–98)	79 (72–81)
$\gamma\delta$ T cells	5 (2–10)	11 (5–16)	5 (2–10)	23 (12–29)
$CD19^+$ B cells	10 (5–13)	52 (48–61)	12 (6–15)	49 (39–62)
$CD25^+$ cells	12 (8–17)	6 (3–11)	21 (18–33)	38 (32–59)

$CD19^+$ and $CD25^+$ cells are expressed as percentages of total lymphocytes and all other subsets as percentages of $CD3^+$ cells.

appendicitis ($P < 0{\cdot}001$, Mann–Whitney two-tailed test) (Fig. 2, Table 1); whereas the appendiceal naive T lymphocyte population was reduced ($P < 0{\cdot}001$, Mann–Whitney two-tailed test) (Fig. 2, Table 1). No significant correlation existed between these changes and either age or gender of the patients (Spearman's correlation). The changes in the peripheral and appendiceal helper T lymphocytes (CD4-positives) and cytotoxic T lymphocytes (CD8-positives) were not significant. (Mann–Whitney two-tailed test) (Table 1).

In the presence of inflammation, there was no significant change in the percentage of peripheral blood $\alpha\beta$ T lymphocyte population. However, there was a significant reduction in the appendiceal $\alpha\beta$ T lymphocyte population when the organ was inflamed ($P < 0{\cdot}001$, Mann–Whitney two-tailed test) (Table 1). Reciprocal changes were observed in the appendiceal $\gamma\delta$ T lymphocyte population ($P < 0{\cdot}001$, Mann–Whitney two-tailed test) (Table 1). The peripheral blood $\gamma\delta$ T lymphocyte population was not affected. There were no significant changes in the B lymphocyte population (Mann–Whitney two-tailed test) (Table 1).

Local arterial and venous lymphocyte subsets

The changes in memory and naive T lymphocytes shown above suggested that memory cells might be sequestered in the inflamed appendix but not in the normal appendix. In the normal appendix, blood taken from the appendiceal artery and vein showed no arterio-venous difference in the percentage of memory cells (Mann–Whitney two-tailed test) (Table 2). In appendicitis, however, the difference was significant ($P < 0{\cdot}001$, Mann–Whitney two-tailed test) (Table 2). Reciprocal changes were observed in the naive T lymphocyte population. In appendicitis, there was an increase in local venous blood naive T lymphocytes as a percentage of total T lymphocytes ($P < 0{\cdot}001$, Mann–Whitney two-tailed test) (Table 2). In the absence of appendicitis, the arterial and venous naive T lymphocyte population were identical (Mann–Whitney two-tailed test) (Table 2).

Peripheral blood and appendiceal lymphocyte CD25 expression

In inflammation, there was an increase in the mean percentage of cells expressing the activation marker CD25 in both

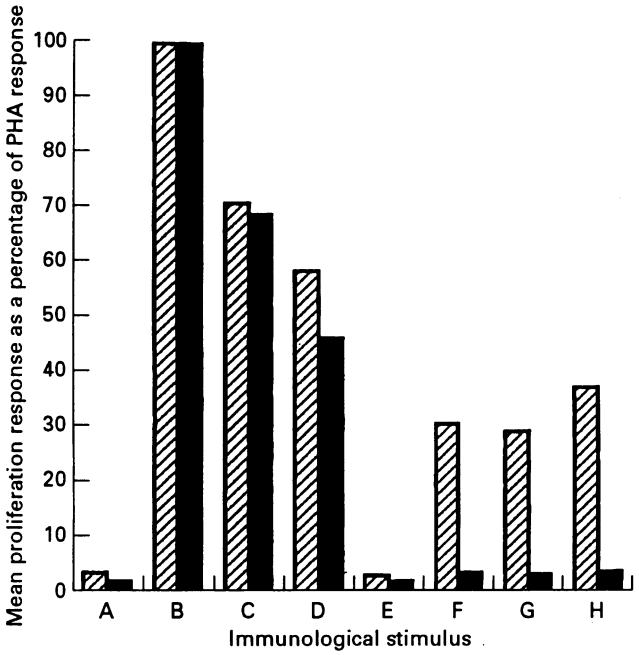

Fig. 3. Mean proliferative responses to mitogens and specific antigens expressed as percentages of the mean phytohaemagglutinin (PHA) response from six patients with normal appendices. ▨, Peripheral blood lymphocytes; ■, appendiceal lymphocytes. A, 48 h control; B, PHA; C, CD2; D, CD3; E, 120 h control; F, PPD; G, TT; H, Flu.

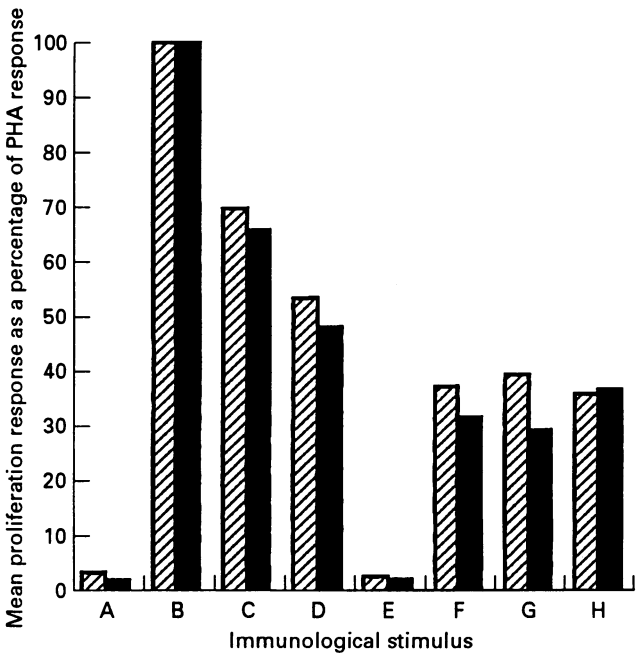

Fig. 4. Mean proliferative responses to mitogens and specific antigens expressed as percentages of the mean phytohaemagglutinin (PHA) response from six patients with appendicitis. ▨, Peripheral blood lymphocytes; ■, appendiceal lymphocytes. A, 48 h control; B, PHA; C, CD2; D, CD3; E, 120 h control; F, PPD; G, TT; H, Flu.

peripheral blood and the appendix (peripheral blood $P < 0{\cdot}05$, Mann–Whitney two-tailed test; appendix $P < 0{\cdot}001$, Mann–Whitney two-tailed test) (Table 1).

Proliferative responses to immunological stimuli

Figure 3 shows the proliferative responses of the peripheral blood and appendiceal lymphocytes to various immunological stimuli. Because the absolute counts incorporated varied quite considerably from experiment to experiment, the results are expressed as a percentage of the PHA response. In a group of six patients with normal appendices, the appendiceal lymphocytes had significantly reduced responses to recall antigens (Flu, PPD and TT) compared with those in the peripheral blood ($P < 0{\cdot}001$, Mann–Whitney two-tailed test). However, in another group of six patients who had appendicitis, the peripheral blood and appendiceal lymphocytes had similar responses to recall antigens (Fig. 4).

Table 2. Mean percentages (range) of lymphocyte subsets, expressed as percentages of $CD3^+$ cells, in appendiceal arterial and venous blood of five patients with appendicitis and five patients with histologically normal appendices

		Arterial	Venous
$CD45RO^+$ T cells	Normal	61 (57–65)	59 (52–63)
	Inflamed	49 (44–55)	38 (33–45)
$CD45RA^+$ T cells	Normal	45 (39–48)	46 (41–49)
	Inflamed	56 (49–60)	67 (62–71)

DISCUSSION

Our results demonstrated the recruitment of lymphocytes to the inflamed appendix. However, the recruitment alone was insufficient to account fully for the peripheral lymphopenia. The reduction in the mean peripheral blood lymphocyte count was $0{\cdot}77 \times 10^9/l$, while the increase in the mean lymphocyte count in the inflamed appendix was $3{\cdot}7 \times 10^9/l$. For an adult with a circulating blood volume of 5 *l*, the inflamed appendix would have to be at least 1·04 *l* in volume to account completely for the peripheral lymphopenia. The entry of lymphocytes into the inflamed appendix therefore did not account for the observed lymphopenia.

Circulating lymphocytes comprise approximately 2% of the total body lymphocyte population [15] and their average circulation time in the peripheral blood is approximately 30 min [16]. The disappearance of lymphocytes from the blood could therefore be due to increased loss from the vasculature or a reduction in their return via veins and lymphatics.

Lymphocytes of all the subsets tested were recruited to the inflamed appendix; but there was a disproportionate increase in memory T lymphocytes, with reciprocal changes in the naive T lymphocytes. This was demonstrated by the changes in memory and naive T lymphocyte subsets in the peripheral blood and appendix (Figs 1 and 2 and Table 2) and further reinforced by the 10% mean shift shown in the naive and memory T lymphocyte subsets between the local arterial and venous blood of the inflamed, but not the normal appendix (Fig. 4). No change occurred in the proportions of peripheral and appendiceal helper or cytotoxic T lymphocyte populations. Changes to the $\alpha\beta$ and $\gamma\delta$ T lymphocyte subsets were confined to the appendiceal population. This may be explained by the local activation and clonal expansion of the $\gamma\delta$ population in

the inflamed appendiceal epithelium, or by activation of $\gamma\delta$ cells in gut-associated lymphoid tissue, which then home rapidly to the inflamed appendix. Since the normal percentage of $\gamma\delta$ T lymphocytes in the peripheral blood was 5%, small changes may not be detectable.

Since the mean percentage of B lymphocytes in the appendix did not alter with inflammation (Table 1) and the absolute number of peripheral blood B cells remained unchanged, it seems likely that local proliferation of B lymphocytes must have occurred in the inflamed appendix. This is not surprising, because the human appendix contains many germinal centres.

Gut T lymphocytes differ from peripheral blood T lymphocytes in having a reduced response to recall antigens [17]. The T lymphocytes harvested from normal appendices all showed a similar lack of response. However, T lymphocytes from the inflamed appendix had very similar recall antigen responses to peripheral blood of the same patients. The recruitment of T lymphocytes to the inflamed appendix appeared dependent on phenotype and not antigen specificity.

CD25 (IL-2R) is a marker of T lymphocyte activation [18]. In appendicitis, there was a percentage increase in CD25 expression in the peripheral blood and in the appendix. Memory and activated T lymphocytes are known to express higher levels of IL-2R than naive T lymphocytes [19]; so it was not surprising to find an increase in the inflamed appendix due to the accumulation of memory T lymphocytes. The increase in peripheral blood is more difficult to explain. It may be due to general activation of the immune system as cytokines and endotoxins are released from the inflamed appendix. Alternatively, these cells may represent lymphocytes activated in the appendix or draining lymph nodes which have entered the blood during recirculation.

Direct evidence for selective recruitment of memory T lymphocytes to inflammatory sites is scarce. Memory T lymphocytes are known to be specifically recruited to skin blisters [20]. Histological observations of other inflamed tissues imply memory cell recruitment to the gut in coeliac disease [21], to the synovial cavity in rheumatoid disease [22], and to the skin in dermatitis [23]. In view of their enhanced expression of activation, adhesion and 'homing' markers, memory T lymphocytes might be expected to be preferentially recruited [24–26]. However, an alternative explanation would be that both naive and memory T lymphocytes enter inflamed sites equally, but become activated and acquire the CD45RO phenotype locally. Our data examining appendiceal arterial and venous blood are the first direct evidence for selective recruitment of CD45RO T lymphocytes to a site of natural inflammation in man. This recruitment was only related to the phenotype and not to antigen specificity. The peripheral lymphopenia associated with this recruitment is a significant sign of sepsis.

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