

## CLINICAL RESEARCH

## Circadian variation of lymphocyte subpopulations: a study with monoclonal antibodies

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### Abstract

Use of monoclonal antibodies to identify subpopulations of circulating lymphocytes in healthy adults showed pronounced circadian variations in total T cells, the two major T cell subsets, and HLA-DR<sup>+</sup> lymphocytes. When the results for the T cell subsets were expressed as a ratio (helper:suppressor) no significant rhythmic variation was observed. Lymphocytes bearing a surface antigen identified by the HNK-1 antibody (a population containing the natural killer and antibody dependent killer activity) did not show significant rhythmic variation. There was an inverse relation between plasma cortisol concentration and numbers of T and B cells.

These observations have therapeutic implications and should be considered in the course of immunological monitoring.

### Introduction

Circadian variation of biological phenomena is often ignored by both doctors and scientists. In this report we describe wide circadian variation of some of the lymphocyte subsets, which

play a part in the development and regulation of the immune response. This study was made possible by the development of a range of well characterised monoclonal antibodies, which in conjunction with flow cytometry permit rapid, precise enumeration of functionally distinct lymphocyte subpopulations.<sup>1 2</sup> As a consequence of these technological developments lymphocyte subpopulations are under intensive study in a wide variety of diseases. For example, alterations of the relative proportions of the T "helper" and T "suppressor/cytotoxic" populations in peripheral blood of recipients of renal allografts correlate with rejection episodes.<sup>3</sup> Since importance is being attached to relatively small changes in the ratio of T helpers to suppressors we considered it important to establish a baseline by investigating physiological variation of the T cell subsets and other subpopulations with a role in the immune response.

TABLE 1—Panel of monoclonal antibodies used

Monoclonal antibody	Cell population identified
Anti-Leu-1 } Anti-Leu-4 }	All T cells
Anti-Leu-3a	Helper subset of T cells
Anti-Leu-2a } Anti-Leu-2b }	Suppressor/cytotoxic subset of T cells
Anti-HLA-DR	B cells and activated T cells (monocytes*)
HNK-1 (Anti-Leu-7)	NK and K cells

\* Monocytes were excluded from analysis using 90° light scatter variables (see text).

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### Methods

Peripheral blood samples were obtained at intervals of three hours from 10 healthy volunteers (mean age 51, range 45-58). Within the constraints of the blood sampling the subjects performed normal daily activities. They slept in a sleep research laboratory, of which they all had previous experience.

Total and differential white cell counts were performed on each blood sample. Plasma was separated and stored frozen for subsequent analysis of cortisol concentrations. Mononuclear cells were isolated on Ficoll Hypaque and then reacted with a panel of monoclonal antibodies to lymphocyte surface antigens (table 1). Mouse monoclonal antibodies

were either conjugated with fluorescein isothiocyanate direct or were visualised with a second, antimouse antibody conjugated with fluorescein isothiocyanate. All analyses were performed on unfixed cell preparations with a fluorescence activated cell sorter (FACS IV, Becton Dickinson FACS Systems, Sunnyvale, California). Ten thousand cells were analysed for each antibody, the number of fluorescent cells being expressed as a percentage of the total lymphocytes. Non-lymphocytic cells contaminating the preparations were excluded from analysis using scatter gates set on the 90° light scatter profile.<sup>3a</sup> The proportions of cells that were fluorescent on analysis with the fluorescence activated cell sorter were used in conjunction with the total and differential white cell counts to calculate absolute numbers of cells in each subpopulation.

Statistical analyses of the percentages and absolute numbers of cells were performed using the methods of Nelson *et al.*,<sup>4</sup> which entailed fitting sine curves to the data from individual subjects and testing whether the variables of these curves showed consistent patterns of circadian variation.

## Results

Variations of all the lymphocyte subpopulations were noted over 24 hours of study. The variations were significant ( $p < 0.05$ ) for absolute numbers of Leu-1<sup>+</sup>, Leu-4<sup>+</sup> (total T cells), Leu-3a<sup>+</sup> (T helper subset), Leu-2a<sup>+</sup>, Leu-2b<sup>+</sup> (T suppressor/cytotoxic subset), and HLA-DR<sup>+</sup> (B cells and activated T cells) lymphocytes. In contrast, HNK-1<sup>+</sup> cells did not exhibit significant circadian variation. Figure 1 shows the variation in the Leu-3a<sup>+</sup> subset of cells, with peak levels occurring at night. When expressed as percentages of the total number of lymphocytes Leu-3a<sup>+</sup> and HNK-1<sup>+</sup> cells showed significant variation.

Plasma cortisol concentration showed the expected large amplitude rhythm and varied inversely with the numbers of T and B cells (fig 2).

The ratio of T helper to suppressor cells (Leu-3a<sup>+</sup>:Leu-2b<sup>+</sup>) showed only minor variations over time and did not exhibit significant rhythmic variation.

Table II summarises the data for each subset.

## Discussion

There is now considerable evidence that the magnitude of the immune response varies with the time of day.<sup>5</sup> The findings of this study may in part explain the variations by demonstrating changes in the number of circulating cells available to encounter and process antigen.

Circadian variation of total lymphocytes,<sup>6</sup> surface Ig<sup>+</sup> cells,<sup>7</sup> and cells forming rosettes with sheep erythrocytes<sup>8</sup> has been reported previously. Our use of monoclonal antibodies in

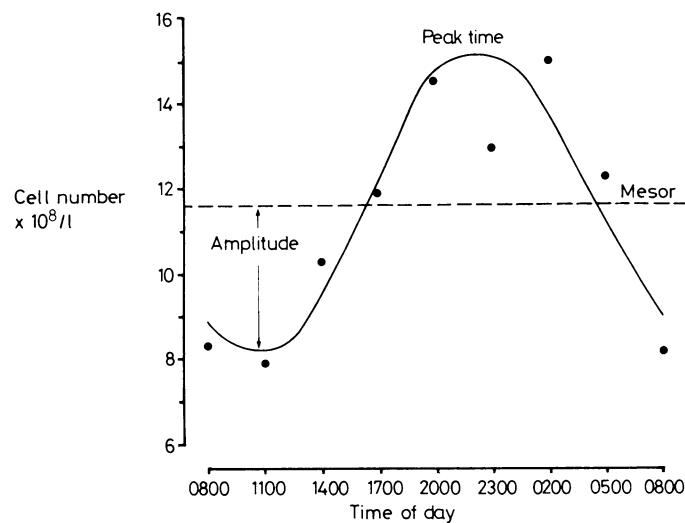


FIG 1—Circadian variation of Leu-3a<sup>+</sup> cells (helper subset of T cells). (Each point represents mean value in 10 subjects.) Mesor 11.66; peak time 2232; circadian variation ( $2 \times \text{amplitude}/\text{mesor}$ ) = 59%.

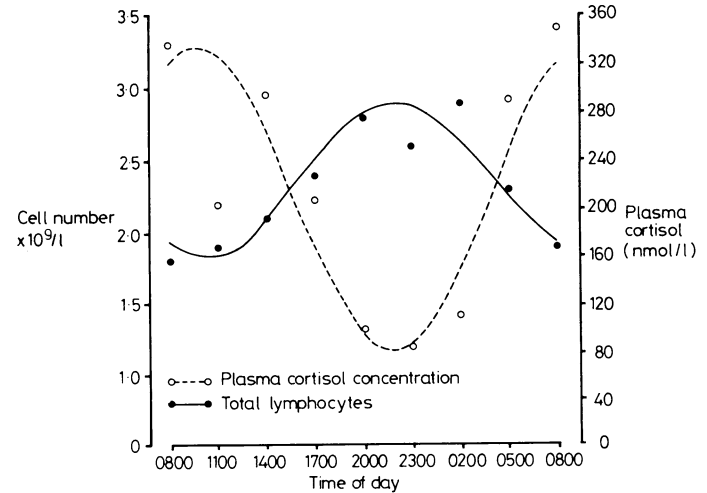


FIG 2—Comparison of circadian variations of total lymphocytes and plasma cortisol concentration. (Each point represents mean value in 10 subjects.) Conversion: SI to traditional units—Cortisol: 1 nmol/l  $\approx$  36.3 ng/100 ml.

TABLE II—Circadian variation of lymphocyte subpopulations contrasted with plasma cortisol variation (data derived from best fitting sine curves)

	Mesor	Amplitude	Peak time	Circadian variation (%)	Significance of circadian rhythm
<i>Results expressed as absolute number of cells <math>\times 10^6/l</math></i>					
Leu-4 <sup>+</sup>	16.0	4.3	2222	54	$p < 0.05$
Leu-3a <sup>+</sup>	11.7	3.4	2232	59	$p < 0.001$
Leu-2b <sup>+</sup>	4.3	1.0	2156	47	$p < 0.05$
HLA-DR <sup>+</sup>	3.5	0.9	2305	53	$p < 0.05$
HNK-1 <sup>+</sup>	2.5				NS
<i>Results expressed as percentage of total lymphocytes</i>					
Leu-4 <sup>+</sup>	66.2				NS
Leu-3a <sup>+</sup>	47.4	5.1	2339	22	$p < 0.05$
Leu-2b <sup>+</sup>	17.9				NS
HLA-DR <sup>+</sup>	14.5				NS
HNK-1 <sup>+</sup>	11.0	2.8	1115	51	$p < 0.01$
<i>Other results</i>					
Helper: suppressor ratio	2.6				NS
Plasma cortisol (nmol/l)	208	126	0948	121	$p < 0.01$

Conversion: SI to traditional units—Cortisol: 1 nmol/l  $\approx$  36.3 ng/100 ml.

conjunction with flow cytometry permitted further subclassification of the lymphocyte population and greater precision in enumeration. It would have been impossible to analyse this number of samples and to count the cells in each sample by light or fluorescent microscopy. The development<sup>9</sup> and characterisation<sup>10-12</sup> of the HNK-1 monoclonal antibody enabled us to identify cells with NK and K cell function by surface labelling, without the necessity for time consuming functional assays.

The variation of numbers of circulating B and T cells points to sizable shifts of cells into and out of the circulation. The traffic of cells through other lymphoid tissue, including bone marrow, may allow for contact between antigen primed regulatory T cells and B cells. The lack of variation of HNK-1<sup>+</sup> cells correlates with the fact that only 0.7% of nucleated bone marrow cells have this phenotype.<sup>10</sup> It also correlates with the finding that in lymphoid tissues HNK-1<sup>+</sup> cells are predominantly located within germinal centres,<sup>12</sup> which are not regarded as part of the lymphocyte recirculation pathway.<sup>13</sup>

The striking inverse relation of the numbers of circulating T and B cells and plasma cortisol concentration, in conjunction with the reported T lymphopenia in patients with Cushing's syndrome<sup>14</sup> and transient lymphopenia after single doses of exogenous corticosteroid,<sup>15,16</sup> suggest a causal interrelation.

The results have certain clinical implications. For example, would the efficiency of antilymphocyte chemotherapy be improved if administration was timed to produce peak plasma

concentrations when the maximum number of cells are in the circulation? Does rejection occur earlier if allografts are transplanted at night, when the number of T cells in the recipients' circulation are at their peak? With respect to the last question, circadian variation in the rejection of rat renal allografts has been described,<sup>17</sup> and the longest surviving grafts were those inserted when the numbers of circulating lymphocytes were at their trough. Sound data relating time of operation to onset of rejection, or graft survival, in man are lacking, although circadian variation in episodes of renal allograft rejection has been suggested.<sup>18</sup>

We have shown that in any attempt at immunological monitoring account must be taken of circadian variation of lymphocyte subpopulations as a source of variance in results. Conversely, we found the ratio of helper to suppressor cells to be fairly stable in our healthy subjects.

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ADDENDUM—Six of the subjects were studied over a further 24 hour period, during which they were deprived of sleep. There were no significant differences in any of the rhythmic variations over this period when compared with the first set of data for these six subjects.

## References

- <sup>1</sup> Reinherz EL, Schlossman SF. Regulation of the immune response-inducer and suppressor T lymphocyte subsets in human beings. *N Engl J Med* 1980;**303**:370-3.
- <sup>2</sup> Herzenberg LA, Herzenberg LA. Analysis and separation using the fluorescence activated cell sorter (FACS). In: Weir DM, ed. *Handbook of experimental immunology*. 3rd ed. London: Blackwell Scientific, 1978:22.1-22.21.
- <sup>3</sup> Cosimi AB, Colvin RB, Burton RC, et al. Use of monoclonal antibodies to

- T cell subsets for immunologic monitoring and treatment in recipients of renal allografts. *N Engl J Med* 1981;**305**:308-14.
- <sup>3a</sup> Ritchie AWS, Gray RA, Micklem HS. Right angle light scatter: a necessary parameter in flow cytofluorimetric analysis of human peripheral blood mononuclear cells. *J Immunol Methods* (in press).
- <sup>4</sup> Nelson W, Tong YL, Halberg F. Methods for cosinorhythmometry. *Chronobiologia* 1979;**6**:305-23.
- <sup>5</sup> Pownall R, Knapp MS. Immune responses have rhythms: are they important? *Immunology Today* 1980 October: vii-x.
- <sup>6</sup> Bartter FC, Delfa CS, Halberg F. A map of blood and urinary changes related to circadian variations in adrenal cortical function in normal subjects. *Ann NY Acad Sci* 1962;**98**:969-83.
- <sup>7</sup> Abo T, Kumagi K. Surface studies of immunoglobulins on human B lymphocytes. III. Physiological variations of SIg<sup>+</sup> cells in peripheral blood. *Clin Exp Immunol* 1978;**33**:441-52.
- <sup>8</sup> Abo T, Kawate T, Itoh K, et al. Characterization of rhythms and cell components in circadian variations of human and mouse lymphocytes. *Chronobiologia* 1979;**6**:71-2.
- <sup>9</sup> Abo T, Balch CM. A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). *J Immunol* 1981;**127**:1024-9.
- <sup>10</sup> Abo T, Cooper MD, Balch CM. Characterization of HNK-1<sup>+</sup> (Leu-7) human lymphocytes. I. Two distinct phenotypes of human NK cells with different cytotoxic capability. *J Immunol* 1982;**129**:1752-7.
- <sup>11</sup> Abo T, Balch CM. Characterization of HNK-1<sup>+</sup> (Leu-7) human lymphocytes. II. Distinguishing phenotypic and functional properties of natural killer cells from activated NK-like cells. *J Immunol* 1982;**129**:1758-61.
- <sup>12</sup> Ritchie AWS, James K, Micklem HS. The distribution and possible significance of cells identified in human lymphoid tissue by the monoclonal antibody HNK-1. *Clin Exp Immunol* 1983;**51**:439-47.
- <sup>13</sup> Ford WL. Lymphocyte migration and immune responses. *Prog Allergy* 1975;**19**:1-59.
- <sup>14</sup> Shohat B, Klein A, Kaufmann H, et al. T lymphocytes and plasma inhibitory factor in ACTH-dependent Cushing's patients. *Journal of Clinical Immunology and Immunopathology* 1979;**13**:452-61.
- <sup>15</sup> Fauci AS, Dale DC. The effect of in vivo hydrocortisone on subpopulations of human lymphocytes. *J Clin Invest* 1974;**53**:240-6.
- <sup>16</sup> Yu DTY, Clements PJ, Paulus HE, et al. Human lymphocyte subpopulations: effect of corticosteroids. *J Clin Invest* 1974;**53**:565-71.
- <sup>17</sup> Ratte J, Halberg F, Kuhl JFW, et al. Circadian variation in the rejection of rat kidney allografts. *Surgery* 1973;**73**:102-8.
- <sup>18</sup> Knapp MS, Cove-Smith JR, Dugdale R, et al. Possible effect of time on renal allograft rejection. *Br Med J* 1979;*i*:75-7.

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# Renal transplantation in children with occult neurogenic bladders drained by intermittent self catheterisation

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## Abstract

Children with neurogenic bladders have usually not been considered for renal transplantation because of the potential complications caused by chronic infections of the urinary tract. Two girls with occult neurogenic bladders who were practising intermittent self catheterisation were given renal transplants. Both took prophylactic antibacterial agents after transplantation, and both experienced infections at some stage, but none of these infections produced a deterioration in renal function. In one patient creatinine clearance fell and signs of

obstruction appeared, but this was because the patient had been catheterising herself only two or three times a week. When she resumed catheterisation four times a day creatinine clearance rose and the urographic and renographic appearances returned to normal.

These results suggest that, with adequate catheterisation and urinary chemoprophylaxis, infection is not a particular problem in children with bladder dysfunction who undergo renal transplantation.

## Introduction

Intermittent clean self catheterisation is an accepted form of managing neurogenic bladders in childhood which does not produce major problems arising from sepsis.<sup>1</sup> It might be supposed that the immunosuppression associated with renal transplantation would lead to problems with infection. We describe two children with occult neurogenic bladders who received renal transplants while practising intermittent self catheterisation after micturition.

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