Clin. exp. Immunol. (1979) **36**, 347–354.

The time of day of antigen encounter influences the magnitude of the immune response

R. POWNALL, P. A. KABLER & M. S. KNAPP *Chronotherapeutics Research Group, Renal Unit, City Hospital Nottingham*

(*Accepted for publication* 20 *November* 1978)

SUMMARY

Studies in the rat and in man have shown that the time of day at which an antigen is encountered has an influence on the expression of any subsequent cell-mediated immunity, when the response is measured after a fixed interval. This suggests that immune processes are modulated by intrinsic biological rhythms. Experiments are now reported in which sensitized rats were ear-challenged with oxazolone* and studied at intervals during the delayed hypersensitivity reaction. These show that differences in the expression of immunity due to the clock time of challenge became apparent at an early stage and were still present after the maximum response, assessed by the change in ear thickness. The experiments also show that the circadian variations in antigen responsiveness are present during the second recall of immunity and can be manipulated by altering the lighting regimen. The light–dark cycle is often a synchronizer for biological rhythms, although its precise role in the oxazolone system remains to be evaluated. This study demonstrates that phase reversal of the lighting regimen alters the proportions of lymphocytes present in rat blood at two clock times, one of which is the time of day at which the maximum immune response to oxazolone is initiated.

INTRODUCTION

Cells involved in delayed hypersensitivity reactions have been studied extensively, as has the time course of their appearance at induced lesions in the skin (Turk, 1975; Jansa, 1977). However, the possibility that cell-mediated immune responses are modified by the time of day of antigen challenge had not been considered prior to our recent studies in the rat (Pownall & Knapp, 1978) and in man (Cove-Smith *et al.*, 1978). This is an important consideration as skin tests are widely used in medical practice, in the evaluation of drugs which may alter the immune response, and in basic immunology.

The study in rats demonstrated a circadian rhythm in delayed hypersensitivity to oxazolone. The response is T cell-mediated (Phanuphak, Moorhead & Claman, 1974), although there are B cells and possible serum components (Zembala & Asherson, 1970). The swelling which results from the use of the ear as the challenge site in immunized animals has been shown to be associated with the arrival of macrophages and oxazolone-specific T cells (Allwood, 1975). When the magnitude of the ear swelling at 24 hr is related to the clock time of the challenge the peak response at 10.00 hr is eight times the lowest response to challenge given at 16·00 hr (Pownall & Knapp, 1978). Similarly, by measuring the area of induration at 48 hr in the forearms of human volunteers we have shown that the mean response to the intradermal injection of tuberculin (purified protein derivative) is over 2½ times greater if the challenge is administered at 07.00 hours than if it is administered at 22.00 hours (Cove-Smith *et al.*, 1978).

Correspondence: Dr R. Pownall, Post Graduate Medical Centre, City Hospital, Hucknall Road, Nottingham NG5 1PB.

* Oxazolone is the abbreviated name for 4-ethoxy-methylene-2-phenyl-oxazolone marketed by British Drug Houses Poole, UK.

0099-9104/79/0050-0347 $02.00 © 1979 Blackwell Scientific Publications

Circadian variations of this magnitude are also evident in immediate type hypersensitivity tests in man, although the maximum and minimum responses observed were at different times from those observed in the cell-mediated immune response (Lee *et al.*, 1977; McGovern, Smolensky & Reinberg, 1977).

The ear assay system in the rat is 100 times more sensitive than conventional skin tests of delayed hypersensitivity (Kostiala, 1977). It has therefore been used to study certain questions raised by our demonstration of circadian variations in cellular immunity. (1) Are the differences in magnitude according to the clock time of challenge present during a second delayed hypersensitivity response? (2) Are the variations evident throughout the development and resolution of the response or are they present only when measurements are made at a fixed interval (24 hr) after challenge? (3) Is it possible to manipulate the pattern of the immune response by an alteration in environmental factors such as phase reversal of the lighting regimen, known to be critical to the phase of many circadian rhythms?

MATERIALS AND METHODS

Rats were sensitized to oxazolone by local application to a shaved area of the abdomen between 10.00 hours and 12.00 hr. The methods of ear challenge and measurement of the immune response by micrometer have been described previously (Pownall & Knapp, 1978).

Three crossover studies, A, B and C, were performed using six groups of rats. Initial ear challenges were given at various clock times in the 24 hr period beginning 9 days after abdominal sensitization. Three groups were challenged at 10.00, 13.00 and 16.00 hours respectively (A, B and C in Table 1) and three groups at 22.00, 07.00 and 04.00 respectively (a, b and c in Table 1). The same rats were challenged again in the 24 hr period beginning 21 days after their initial sensitization but at their appropriate crossover times (Table 1). In other animals, groups D, E and F, the repeat challenge was given at the same clock time (Table 1), that at 10.00 hours being given 10 and 22 days after sensitization.

In all cases the group size was six and the data reported are the differences (group mean$\pm$s.e.m. in μm) between the initial measurement of the responding ears and those determined exactly 24 hr later without interference during this period. Illumination was from 10.00 hr to 22.00 hr alternating with complete darkness (schedule 1).

Two further groups each of nine rats on the illumination regimen of Schedule 1 were challenged on day 9 at 10.00 hr and 22.00 hr respectively. Their responses were determined every 12 hr for 48 hr. One week later the light : dark phases were abruptly reversed with darkness from 10.00 hr to 22.00 hr alternating with light (schedule 2). After stabilization for 5 weeks on the new regimen, the second challenge was administered at the same clock times as the initial day 9 challenge. Measurements were again obtained at 12 hr intervals for 48 hr.

Individual blood samples were obtained commencing at either 10.00 hr or 22.00 hr from two groups each of nine rats maintained on light schedule 1. Blood was taken from the heart, or the junction of the iliac artery with the aorta, using

TABLE 1. The experiment design for the crossover and repeat studies

	Groups/time in crossover						Groups/time in repeat		
	A	a	B	b	C	c	D	E	F
Initial challenge*	10.00	22.00	13.00	07.00	16.00	04.00	04.00	10.00	16.00
Re-challenge†	22.00	10.00	07.00	13.00	04.00	16.00	04.00	10.00	16.00
Change in clock time (hr)	12		18		12		0	0	0

* Day 9 (except E) when day 10.

† Day 21 (except E) when day 22.

Three crossover studies were performed pairing clock times 10.00 and 22.00 hr in A, 13.00 and 07.00 hr in B, and 16.00 and 04.00 in C. Ear challenges were carried out in the six groups of rats A, a, B, b, C and c at different clock times during the 24 hr period 9 or 21 days after initial sensitization to oxazolone. In groups D, E and F the first and second challenges were repeated at the same clock times.

sodium pentobarbitone anaesthesia (60 mg/kg). The procedure was repeated in groups of different rats maintained for over 2 months on the reversed lighting regimen of schedule 2.

Data were analysed by analysis of variance techniques appropriate to the fully balanced crossover design, or by the *t*-test, pairing values from the same animal when appropriate (Smart, 1970).

RESULTS

Measurements of immune responses can be compared to the increases in ear thickness observed in control groups, each made up of six non-sensitized rats maintained on schedule 1. Oxazolone application at 10.00 hr and 22.00 hr resulted in 24 hr increments of 16±9 μm and 7±5 μm respectively. At these times the 24 hr changes due to the application of turpentine oil, a non-specific irritant, were 1±4 μm and 15±3 μm respectively. In rats immunized to oxazolone the changes in the ear which is not challenged with oxazolone are of a similar low order of magnitude (Pownall & Knapp, 1978).

Data from the crossover studies are illustrated in Fig. 1 and the results of statistical analyses presented in Table 2. In all three crossover experiments the first challenge had no apparent influence on the magnitude of the second challenge. The magnitudes of the immune responses at 10.00 hr and 04.00 hr were very different from those seen at their respective crossover times, namely 22.00 hr and 16.00 hr. Therefore, in crossovers A and C the effect of changing the time of challenge on the magnitude of the response was significant at the 0·1% level. This significance was lower in crossover B ($P<0{\cdot}05$) which might be anticipated as the magnitudes of the responses at 13.00 hr and 07.00 hr were more nearly comparable (Fig. 1). Also, in crossover B this effect and the use of the fully balanced crossover design resulted in the

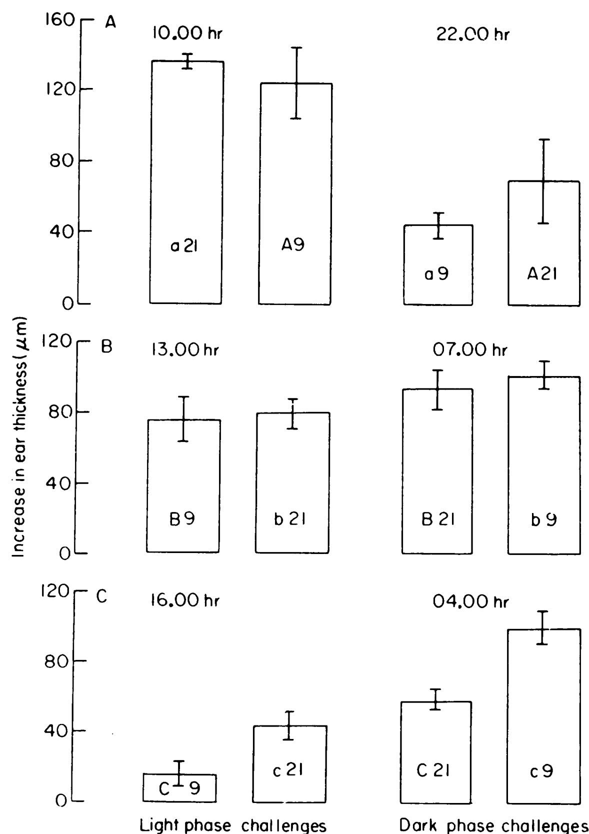

FIG. 1. Mean±s.e. increases in ear thickness (μm) 24 hr after oxazolone challenge in three crossover studies: (A) between 10.00 and 22.00 hr; (B) between 13.00 and 07.00 hr; and (C) between 16.00 and 04.00 hr. Within the blocks, the letters identify the groups, each of six rats, and the numbers indicate ear challenges 9 or 21 days after initial sensitization.

TABLE 2. Analysis of variance of the results of the crossover trials illustrated in Fig. 1

Source of variation	Degrees of freedom	Crossover A (10.00 × 22.00) Sum of squares	Mean square	F*	P	Crossover B (13.00 × 07.00) Sum of squares	Mean square	F	P	Crossover C (16.00 × 04.00) Sum of squares	Mean square	F	P
Groups	1	267	267	0·2262	n.s.†	287	287	0·287	n.s.	6700	6700	13·987	0·01
Between rats within groups	10	11,818	1182	0·6346	n.s.	9984	998	5·015	0·01	4786	479	2·768	n.s.
Clock time of challenge	1	32,708	32,708	17·5655	0·01	1786	1786	8·974	0·025	13,207	13,207	76·341	0·001
1st/2nd challenge	1	1701	1701	0·9133	n.s.	2·4	2·4	0·01	n.s.	187	187	1·080	n.s.
Remainder	10	18,621	1862	—	—	1991	199	—	—	1725	173	—	—
Total	23	65,116	—	—	—	14,050	—	—	—	26,605	—	—	—

* Variance ratio (see Smart, 1970).
† Not significant.

TABLE 3.

Clock time	1st challenge	2nd challenge	P
16.00 hr	28·6 ± 7·8	40·2 ± 12·5	n.s.
04.00 hr	78·5 ± 9·9	45·6 ± 7·7	0·01
10.00 hr (day 10)	140·6 ± 7·2	129·2 ± 7·3	n.s.

Mean ± s.e. increases in ear thickness (μm) in groups of six rats. The first and second challenges were given at the same clock times.

'between rats within groups' becoming significant at the 1% level (Table 2) which indicated a significant reduction in the error variance (Smart, 1970).

The results in Table 3 were obtained from rats in which the challenges were repeated at the same clock times. The first and second responses at 16.00 hr or 10.00 hr (day 10) were similar ($P > 0·05$, paired t-test). The magnitudes of these responses were also comparable to those seen in different rats in the appropriate crossed over groups, A,a, at 10.00 hr and C,c, at 16.00 hr. The magnitude of the response is therefore reproducible at these clock times. The second response to repeated challenge at 04.00 hr is, however, lower than the first ($P < 0·01$) when the challenges are repeated at this clock time (Table 3). This result at 04.00 hours might be expected to be different if the crossover results for a second challenge at this time are considered, as the significance of the group variation in crossover C arises mainly from the failure of the group being challenged for the second occasion at 04.00 hr (group C21 in Fig. 1) to respond to the same extent as the group challenged initially at this clock time (group c9 in Fig. 1).

The effect of reversing the light : dark cycle on the development of the immune response is shown in Fig. 2. The groups were challenged at the same clock times, either 10.00 hr or 22·00 hr on the first and second occasions. The results show that the measured response was at a maximum 24 hr after challenge.

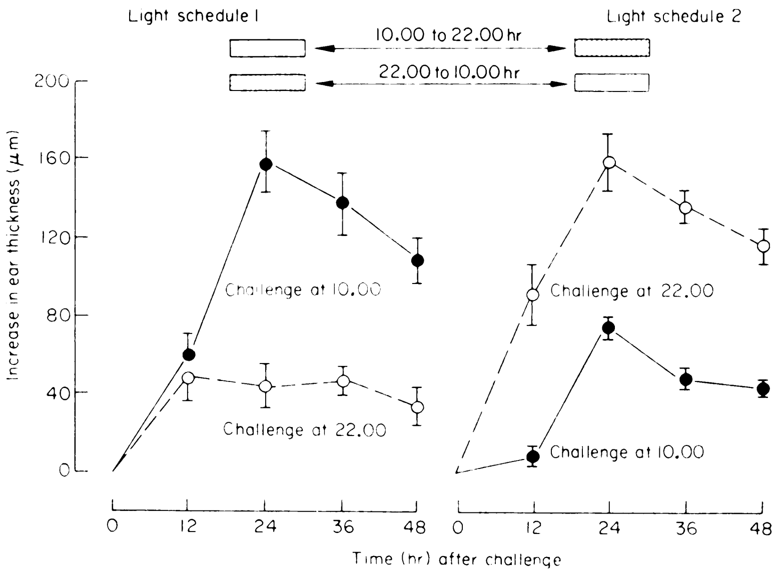

FIG. 2. Mean ± s.e. increase in ear thickness (μm) in groups of nine rats measured at 12 hr intervals after oxazolone challenge. The groups were challenged at 10.00 hr (●) or 22.00 hr (○) for the first time on light schedule 1. The light phase was then shifted by 12 hr, and the rats were rechallenged at the same clock times on light schedule 2.

Again, the responses on schedule 1 were comparable to those seen in the crossover and repeat trials in which measurements were only made 24 hr after challenge. Apparently, the initial clock time of the challenge influences the subsequent magnitude of the response since on both lighting regimens the difference between the groups at 24 hours was maintained to 48 hours ($P < 0{\cdot}005$). These differences between groups did not become evident until 24 hr on light schedule 1 ($P < 0{\cdot}001$), whereas on schedule 2 the group difference was evident in the measurements taken 12 hr after challenge ($P < 0{\cdot}005$).

The responses of the group challenged initially at 10.00 hr during the light phase were lower following the second challenge at the same clock time, but on this occasion challenged during the dark phase of the reversed lighting regimen ($P < 0{\cdot}005$ for the paired *t*-tests at all intervals after challenge). Similarly, the responses of the group challenged on both occasion at 22.00 hr were reversed by altering the lighting regimen. Twelve hours after challenge the response was significantly higher on schedule 2 than on schedule 1 ($P < 0{\cdot}005$) and the responses measured at 24, 36 and 48 hr remained significantly higher ($P < 0{\cdot}001$) than those seen during the first challenge in this group of rats. It is clear therefore that the effect of the 12 hr phase shift in the lighting regimen of both groups of rats was to bring about an inversion of the pattern of their immune response to challenge at either 10.00 hr or 22.00 hr.

In rats which were not undergoing an immune response to challenge with oxazolone, there were more lymphocytes present in the blood at 10.00 hr than at 22.00 hr on light schedule 1 (Table 4). When the illumination phase was shifted by 12 hr, it was found in other rats that the difference in lymphocyte numbers at these clock times was also reversed so that in the rats on schedule 2 there were greater numbers of lymphocytes at 22.00 hr.

TABLE 4. Rat lymphocyte counts at 10.00 and 22.00 hr

Sample time (hr)	Light schedule 1	Light schedule 2
10.00	4107±304	3431*±337
22.00	2036±165	6191±370
P	0·001	0·001

Rat lymphocyte counts (mean±s.e.m. per mm^3) observed at either 10.00 or 22.00 hr in different groups of nine rats maintained on schedule 1 (light 10.00 to 22.00, then darkness) or schedule 2 (light 22.00 to 10.00, then darkness). One rat with a lymphocyte count of 10,430, indicating a current infection, has been excluded from the group marked with an asterisk. Its inclusion reduces the significance level between groups on schedule 2 to 5%.

DISCUSSION

The elevated levels of blood lymphocytes which were observed in nocturnally active rats at the end of the dark phase of either illumination schedule presumably correspond to similar circadian changes which have been shown to occur in the human where the lymphocyte count rises during daytime activity (Tavadia *et al.*, 1975), or following a period of exercise (Steel, Evans & Smith, 1974). The greater number of lymphocytes on both light schedules coincides with the clock time of the maximum observed immune response. This observation suggests a possible role for lymphocyte availability in any explanation of the differences of the challenge. Their presence in greater numbers, either locally or centrally (as is evident in the blood), may result in an increased likelihood of their encountering and processing an antigen. The converse of this mechanism has been put forward as one mechanism by which corticosteriods act as immunosuppressants. Following the administration of cortisone acetate, lymphocytes are redistributed to the bone marrow, resulting in lymphocytopoenia (Fauci, 1975). It is possible that the balance between the numbers of lymphocytes sequestered elsewhere, in relatively inaccessible sites such

as the bone marrow, or retained in the thymus (Davies, 1969), affects the immediate transport of lymphocytes to the site of an immune challenge at a particular time. This in turn might produce circadian variations in the aggregation of the responding cells at the site of an immune challenge measured as the degree of swelling after a fixed interval.

Oxazolone probably remains at the ear as an antigenic stimulus for at least 48 hr, having combined with skin protein. A circadian rhythm in the mitotic activity of the ear epidermis has been observed (Rensing & Goedke, 1976) and circadian variation in the processing of low molecular weight haptens, like oxazolone, might affect their rate of presentation to the cells of the immune system. Neither this hypothesis, nor that advanced earlier concerning the availability of lymphocytes seems to be the complete explanation of the pattern of response seen in Fig. 2. As the antigen persists at the site of challenge, it seems reasonable to postulate that any response might be similar to that observed in the early stages of the response, but that the 'handing over' of the antigen to available lymphocytes in the second 24 hr span would result in the responses catching up with one another. By 48 hr the responses observed might be more nearly comparable. This does not happen (Fig. 2). The clock time at which the hapten is applied to the ear, or a sequence of events initiated close to that time, determines the magnitude of the subsequent immune response throughout the whole of the 48 hr period studied.

Although delayed hypersensitivity responses have been studied extensively there is as yet no reported investigation in which the responding cells have been assessed qualitatively or quantitatively according to the clock time of their arrival at the site of an immunological insult. The oxazolone response needs to be investigated at the cellular level to elucidate the factors in the immune system which bring about the observed circadian variations in the immune response. Perhaps suppressor T cells (Asherson & Zembala, 1975), or the interactions of the T cell, B cell, macrophage and serum components of the oxazolone response (Zembala & Asherson, 1970) are modulated at different levels during a 24 hr span. Alternatively, or additionally, challenge at a particular time of day may favour the proliferation of a particular class of lymphocyte because at, or soon after, challenge that population of cells may be closer to the mitotic stage of their cell cycle. The endocrine content of the blood which is known to be rhythmic (Philippens, von Mayersbach & Scheving, 1977) may be relevant to the response.

The demonstration of rhythms in cell-mediated immunity, as well as in humoral immunity (McGovern *et al.*, 1977) has profound implications for the way in which such studies are conducted. The efficacy of drugs which affect immune processes cannot be evaluated unless rhythmic variations in the activity of those processes have been either discounted or fully evaluated. The findings reported in this paper suggest that studies of biological rhythms in immunological events and of the influence of other biological rhythms on these events may help to increase the understanding of control mechanisms in immune reactions, and may contribute to an understanding of disturbances of the immune processes.

This study was financed by the National Kidney Research Fund and the Trent Regional Health Authority. The authors are grateful to many people who have given help and advice, particularly Dr T. Wallington and Dr G. Reeves, Immunology Department, University Hospital, Nottingham. Special thanks are due to D. Byrne of the Haematology Department, City Hospital who undertook the lymphocyte counts, and the staff of the animal laboratories, University of Nottingham Medical School.

REFERENCES

ALLWOOD, G.G. (1975) The migratory behaviour of T blasts to contact sensitivity reactions in actively and passively sensitized mice. *Immunology,* **28,** 681.

ASHERSON, G.L. & ZEMBALA, M. (1975) Inhibitory T cells. *Curr. Top. Microbiol. Immunol.* **72,** 55.

COVE-SMITH, J.R., KABLER, P., POWNALL, R. & KNAPP, M.S. (1978) Circadian variation in an immune response in man. *Br. Med. J.* ii, 253.

DAVIES, A.J.S. (1969) The thymus and the cellular basis of immunity. *Transplant Rev.* **1,** 43.

FAUCI, A.S. (1975) Mechanisms of corticosteroid action on lymphocyte subpopulations. I. Redistribution of circulating T and B lymphocytes to the bone marrow. *Immunology,* **28,** 669.

JANSA, P. (1977) Cellular evaluation of hypersensitivity to 2, 4-dinitrochlorobenzene. *Neoplasma,* **24,** 529.

KOSTIALA, A.A.I. (1977) Radiometric ear index test as a measure of delayed-type hypersensitivity in the rat. *Immunology,* **33,** 561.

LEE, R.E., SMOLENSKY, M.H., LEACH, C.S. & MCGOVERN,

J.P. (1977) Circadian rhythms in the cutaneous reactivity to histamine and selected antigens, including phase relationship to urinary cortisol excretion. *Ann. Allergy,* **38**, 231.

McGovern, J.P., Smolensky, M.H. & Reinberg, A. (1977) *Chronobiology in allergy and immunology.* Charles C. Thomas, Springfield, Illinois.

Phanuphak, P., Moorhead, J.W. & Claman, H.N. (1974) Tolerance and contact sensitivity to DNFB in mice. I. *In vivo* detection by ear swelling and correlation with *in vitro* cell stimulation. *J. Immunol.* **112**, 115.

Philippens, K.M.H., von Mayersbach, H. & Scheving, L.E. (1977) Effects of the scheduling of meal-feeding at different phases of the circadian system in rats. *J. Nutr.* **107**, 176.

Pownall, R. & Knapp, M.S. (1978) Circadian rhymicity of delayed hypersensitivity to oxazolone in the rat. *Clin. Sci. Mol. Med.* **54**, 447.

Rensing, L. & Goedeke, K. (1976) Circadian rhythm and cell cycle: possible entraining mechanisms. *Chronobiologia,* **3**, 53.

Smart, J.V. (1970) *Elements of medical statistics.* Staples Press, London.

Steel, C.M., Evans, J. & Smith, M.A. (1974) Physiological variation in circulating B cell: T cell ratio in man. *Nature (Lond.),* **247**, 387.

Tavadia, H.B., Fleming, K.A., Hume, P.D. & Simpson, H.W. (1975) Circadian rhythmicity of human plasma cortisol and PHA-induced lymphocyte transformation. *Clin. exp. Immunol.* **22**, 190.

Turk, J.L. (1975) *Delayed hypersensitivity*, 2nd edn, p. 53. North-Holland, Amsterdam.

Zembala, M. & Asherson, G.L. (1970) Contact sensitivity in the mouse. 5. The role of macrophage cytophilic antibody in passive transfer and the effect of trypsin and antigammaglobulin serum. *Cell Immunol.* **1**, 276.