When Should the Immune Clock Be Reset?

From Circadian Pharmacodynamics to Temporally Optimized Drug Delivery

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INTRODUCTION

Through its permanent interactions with the environment, the immune system constitutes one the major sources of external information of the whole organism. Immune cells are disseminated throughout all parts of the body, and notably in those exposed to environmental stimuli (skin and gut mucosa). Following exposure to a chemical agent, these cells assess whether this molecule belongs to "self" or to "nonself" ("immune surveillance").[1] Most often, macrophages first take up and "analyze" these foreign substances. These cells bear major histocompatibility complex (class II) molecules at their surface and will either destroy the chemical or present it as an antigen to T lymphocytes. Subsequently various cytokines will be secreted and several lymphocyte subsets will cooperate for mounting the immune response. Two goals will be aimed at: to instruct lymphocytes to become cytotoxic for all cells that bear this antigen at their surface and to learn how to quickly recognize and destroy them (so-called K and cytotoxic T lymphocytes). NK lymphocytes do not require such a process and are directly cytotoxic, primarily for tumor cells. Other leukocytes, such as polymorphonuclear cells, are also involved in the clearing of foreign cells or necrotic materials from the body.

The immune system is thus indispensable for the individual's life. So may be the temporal organization of biologic functions along several time scales.[2] Endogenous and genetically based rhythms are thought to result from an adaptative process of living species to environmental cycles.[2]

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In 1987, W. E. Paul, in his presidential address to the American Society of Immunology, emphasized that the coming century would be that of the understanding of the physiology of the immune system.[3] In this article, we propose and discuss an original model which depicts the coordination of the several immune functions along the 24-h scale in man. The results that will be stressed indicate that immune functions of living beings are highly and predictably organized in time. As a result, (1) the immune response to antigen presentation differs both quantitatively and qualitatively according to time of exposure; (2) the pharmacodynamic effects of substances that affect immunity ("biological response modifiers," BRM) strongly and predictably depend upon dosing time (clock hour, stage of the menstrual cycle, season, . . .); (3) chronotherapy (a strategy aimed at delivering the amount of drug needed at appropriate times) should become a necessary step for fully exploiting the pharmacologic properties of BRMs. Clini-

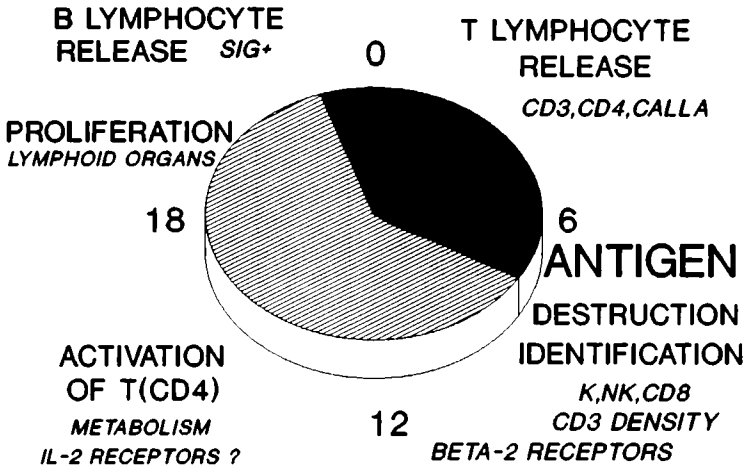

FIGURE 1. The immune circadian clock of man. This proposed model applies to healthy adult human subjects, synchronized by daily activities (from 0700 h to 2300 h) and nocturnal rest (from 2300 h to 0700 h).

cal data will be complemented by results from animal experiments whenever needed.

CIRCADIAN-ADAPTED IMMUNE SURVEILLANCE

The normal coordination of several human immune functions along the 24-h time scale is tentatively depicted in FIGURE 1.

This original hypothetical model is based upon both our own data and an extensive literature review. It was felt as timely to synthetize our knowledge on the human immune clock. This model should help to generate and test physiologic, physiopathologic, and pharmacologic hypotheses and guide studies of the interplay of such an immune circadian clock with other biological clocks.

Antigen Recognition and Destruction

From an adaptative viewpoint, the identification of a foreign substance and its subsequent destruction constitute the main task of the immune system. Such functions appear greater in the second half of the night or in the morning in diurnally active subjects. Human data on antigen recognition are scarce, and experiments have seldom addressed this very initial stage of immune defense. Nonetheless, the skin response of sensitized subjects to tuberculin challenge was 2.5-fold larger following antigen exposure at 0700 h as compared to 2200 hrs,[4] and episodes of rejection of kidney allograft were estimated to be triggered at 0600 h in patients bearing a renal transplant.[5]

In nocturnally active mice, macrophage reactivity to a phagocytic stimulus as gauged by zymosan-induced chemiluminescence was greatest in the middle to late rest span[6–8] or early activity span.[8] Other phagocytic cells, such as polymorphonuclears, had increased migratory activity at these times.[9]

Cytotoxic cells are either antigen specific cytotoxic T lymphocytes [which bear the CD8 epitope at their surface—T (CD8+)] or nonspecific lymphocytes; killer (K) and natural killer (NK) cells respectively destroy cells coated with immunoglobulin (Ig) G and some tumor cells. FIGURE 2 displays the circadian rhythms that characterize these cells.[10–14] Murine data indeed indicate that the organism is more resistant to tumor cells in the middle to late rest span[15,16] when an increased number of NK lymphocytes are circulating[17] (and unpublished data, FIGURE 3). Results shown in FIGURES 2 and 3 emphasize that immune data obtained in nocturnally active laboratory rodents such as mice or rats usually correspond well to human data, if referred to the species-specific rest-activity cycle.

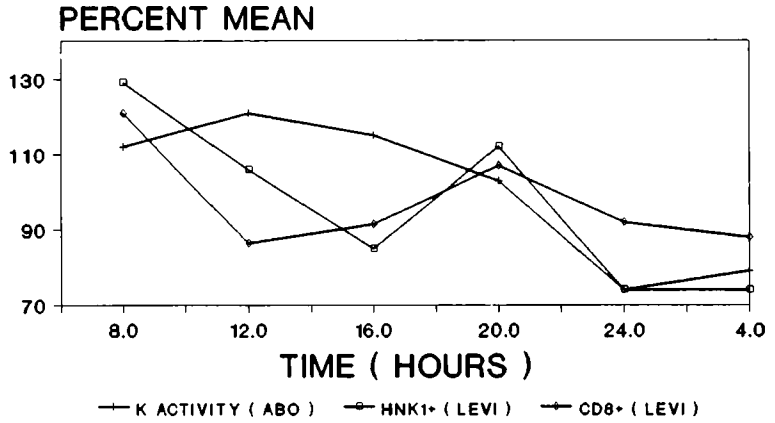

FIGURE 2. Circadian rhythms in circulating cytotoxic cells of healthy young adults. Peak occurred at 0800 h or at 1200 h, with a marked depression in the early night. After Abo *et al.*[10] and Lévi *et al.*[13,41]

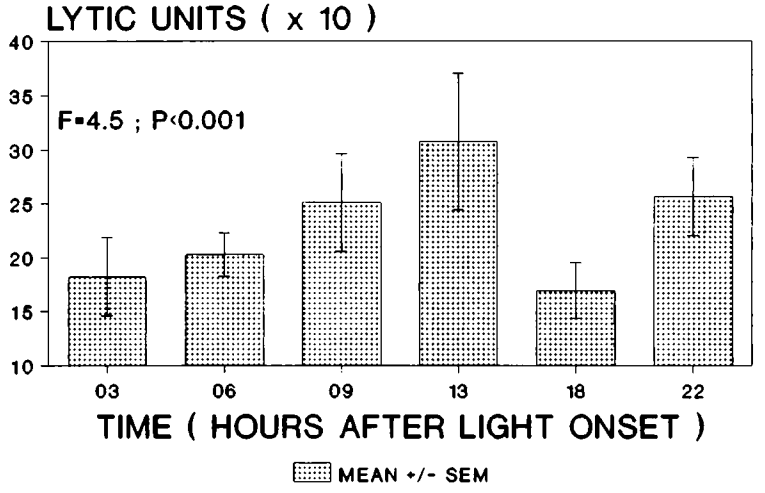

FIGURE 3. Circadian rhythm in NK cell activity of murine splenocytes (B6D2F1 male mice aged 9–10 weeks). NK activity, as gauged against YAC-1 tumor cells, was twice higher at the beginning of the active span of mice (13 hours after light onset, or HALO) as compared to mid activity (18 HALO) or early rest (3 HALO).

T Cell Response

In order for an immune response to be elicited, the antigen is usually presented by macrophages to T lymphocytes. Such interaction occurs via the T-cell receptor. The latter is intimately related to a glycoprotein molecule called CD3 (third cluster of differentiation), which characterizes T lymphocytes [T (CD3+)] and is recognized with anti-CD3 monoclonal antibodies. The presence of CD4 molecules on the surface of T (CD3+) lymphocytes makes these cells able to further amplify the immune response [helper T lymphocytes, T (CD4+)]. Such a response will be primarily B or T mediated.

The density of CD3 molecules at the surface of T lymphocytes was two- to threefold higher in the morning than in the evening or early night (FIGURE 4). Similar results were obtained for the density of CD4 molecules on the surface of T (CD4+) lymphocytes.[18] This indeed suggests that the availability of T cell receptors for antigens is increased at that time, so that the efficiency of T cell response is maximized then. Results from lymphocyte exposure to lectins such as phytohemagglutinin (PHA) support this hypothesis. Lectins are cell recognition molecules,[19] which are nonspecific inducers of lymphoblastic transformation and proliferation. The response of lymphocytes to PHA closely resembles that elicited by antigen presentation, and only involves T cells. Proliferation of T cells was twice as large following PHA exposure of separated lymphocytes in the early morning (0600–1000 h) as compared to late evening (1800–2200 h).[20,21] When whole blood,

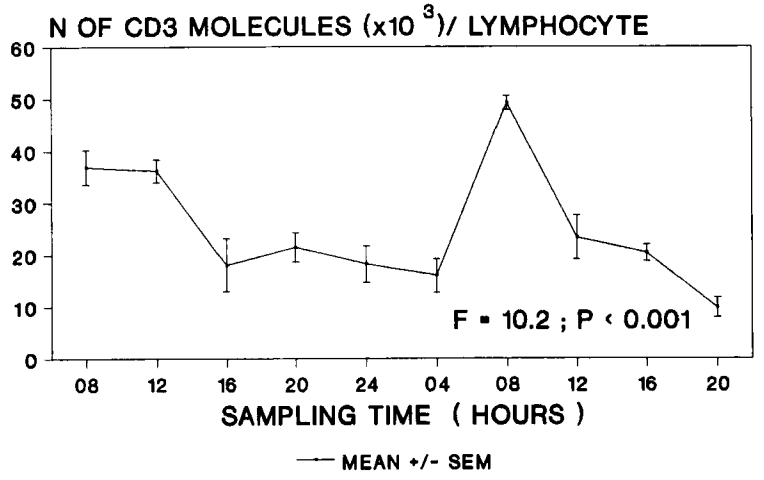

FIGURE 4. Circadian rhythm in the density of CD3 molecules on human lymphocyte surface. Data obtained in 5 healthy subjects, active from 0700 h to 2300 h, and recumbent at night. Blood samples were obtained every 4 h. The density of these molecules was assessed by flow cytometry. After Canon *et al.*[18]

rather than mononuclear cells, was exposed to PHA, greatest lymphoblastic transformation occurred at 2000 h.[21,22]

An explanation for such a discrepancy has been provided.[23] T lymphocytes were more prone to proliferate following contact with autologous PHA-stimulated T cells at 0800 h than at 1200 or at 2000 h. The reverse was true if T lymphocytes were exposed to non-T cells. This indeed documents that a rather precise coordination in time characterizes these different stages of immune defenses.

T Cell Activation

After antigen–T cell receptor interaction, T cells become activated, as indicated by the following events: increased intracellular potassium and calcium concentrations, activation of membrane methyltransferases, adenylate and guanylate cyclases, enhanced membrane fluidity, RNA synthesis, expression of interleukin-2 (Il-2) receptors on the T lymphocyte surface. A physiologic state of latent activation seems to characterize T lymphocytes between 1200 and 1800 h. Thus energy metabolism, as gauged by several enzyme activities, was highest at 1600 h in circulating lymphocytes.[24] A peak in RNA content of total lymphocytes was observed at 1800 h, another one occurring at 0600 h.[25] Low-affinity interleukin-2 receptors tended to be expressed in a significantly larger number of circulating lymphocytes (CD25+) in the early afternoon, in healthy subjects. However, spontaneous expression of these receptors was very low as expected.[18] In human erythrocytes, the fluidity of the cell membrane was increased at 1800 h, the activity of methyltransferase I was highest at 1800–2400 h, and intracellular potas-

sium concentration was greatest at 1800 h (FIGURE 5).[26] Similar circadian oscillators might govern these rhythms in different blood cells.

Lymphocyte Proliferation

Proliferation of immune cells primarily occurs in lymphoid organs. B cells are located mostly in bone marrow but also in the germinal centers of lymph nodes

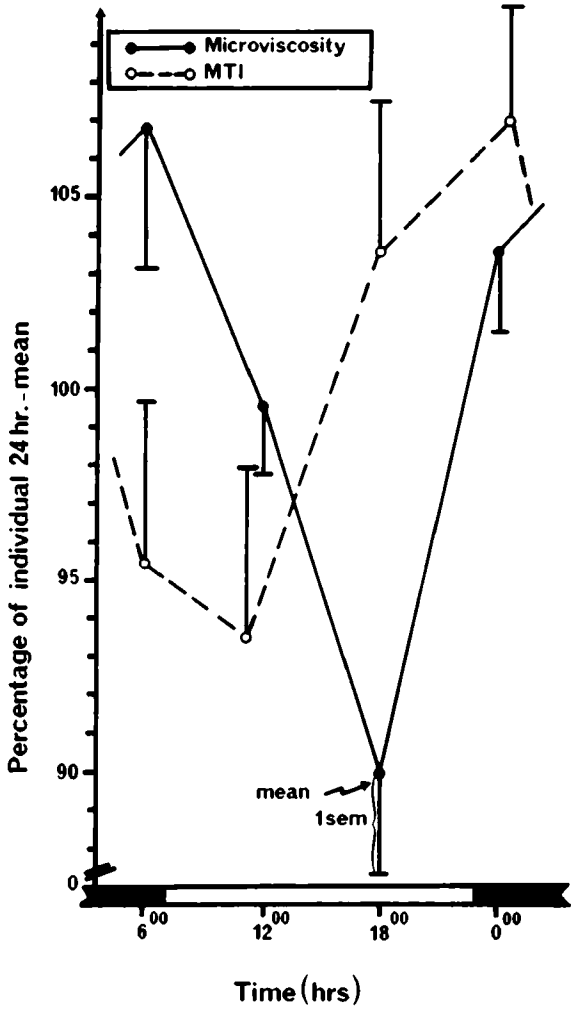

FIGURE 5. Circadian rhythms in membrane erythrocytes. Blood was obtained every 6 h for 24 h in 7 diurnally active healthy subjects. Membrane fluidity was highest at 1800 h ($p < 0.001$) and negatively correlated with the activity of membrane-bound methyltransferase I (MTI) ($p < 0.001$). After Lévi *et al.*[26]

and in spleen; T cells proliferate and differentiate mostly in thymus in the early stages of life, then do so in the deep cortex of lymph nodes and in spleen.

Cell proliferation, as gauged by DNA synthesis, was highest near 1600–2000 h in human bone marrow.[27] The proliferative ability of bone marrow granulomonocytic precursors was also highest in the second half of the activity span both in man[27] and in mice.[28] These cells give rise to phagocytic cells, namely, polymorphonuclear cells, monocytes, and macrophages. Maximal thymic DNA synthesis also occurred in the late activity span of mice[29,30] or guinea pigs.[31] Nonetheless, results obtained in mice may differ from one strain to another since these circadian rhythms have a genetic basis.[32,33] Human peripheral blood lymphocytes also exhibited a spontaneous circadian variation in DNA synthetic activity, with two peaks, one in the morning (0800–1000 h), the other one in the evening (2000–2400 h) with a marked depression at 0400 h.[22,34]

Lymphocyte Blood Release

Lymphoid organs will subsequently release lymphocytes in peripheral blood: the counts of circulating total lymphocytes have long been known to be 50–100% higher in the early night (midnight–0400 h) than in the morning (0800–1200 h).[2,10,12–14,20–24,34–37] B lymphocytes seem to be released between 2000 and 2400 h, a few hours before T cells (between 2400 and 0400 h).

Mature B lymphocytes can be characterized by surface immunoglobulins (Ig) and identified by a monoclonal antibody directed against Ig (SIg+). The circulating count of B cells doubled between 0800 h and 2000 or 2200 h[10,35] (FIGURE 6). Different laboratory techniques and study designs and/or infradian periodicities may account for discrepant results in one study[21] as discussed elsewhere.[36] The

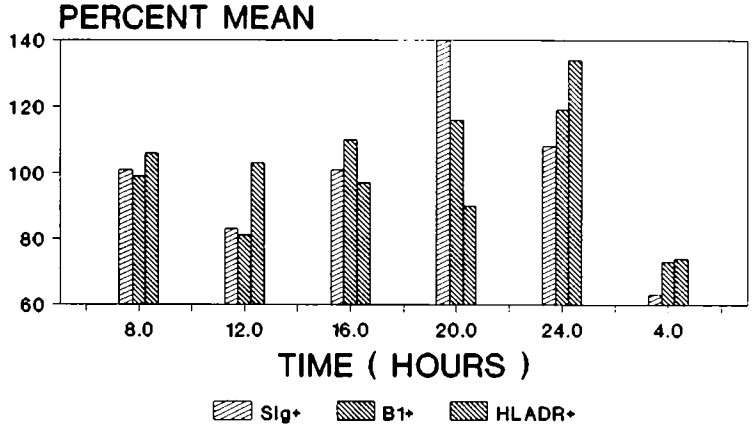

FIGURE 6. Circadian changes in circulating B lymphocytes of diurnally active healthy young adults. Mature (SIg+ and B1+) and activated (HLA-DR+) B cells are respectively highest near 2000 h and near 2400 h. Data obtained in 7 subjects. After Lévi *et al.*[35] and Canon *et al.*[44]

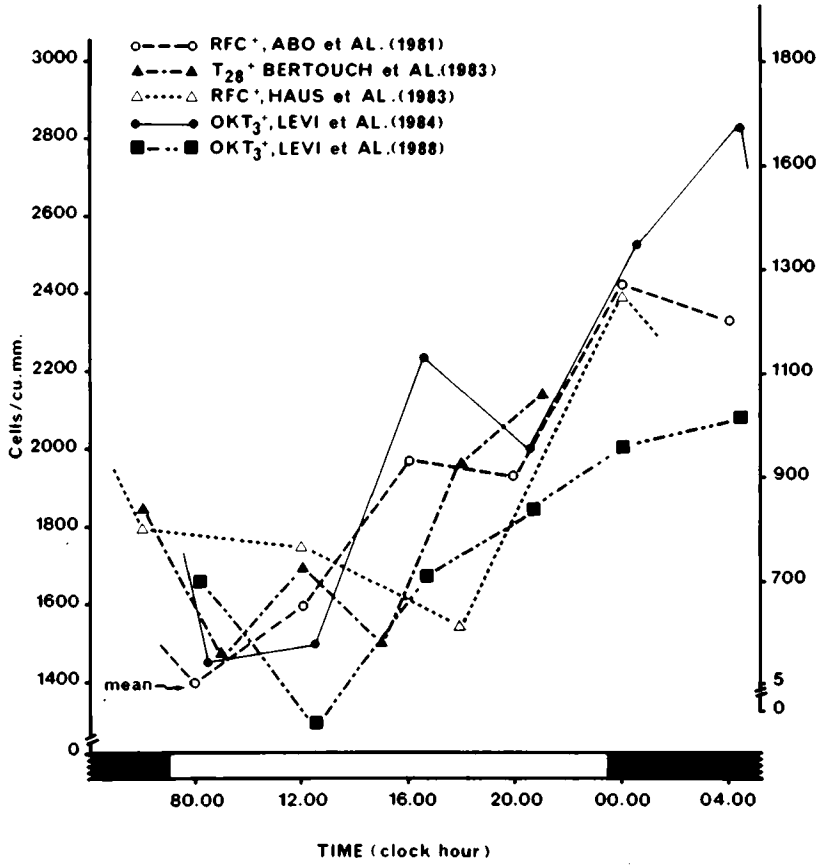

FIGURE 7. Circadian rhythm in circulating T-lymphocytes in healthy human beings. Note reproducibility of results despite different methods, subjects, and geographic locations. After Abo *et al.*,[10] Haus *et al.*,[21] Lévi *et al.*,[35,41] and Bertouch *et al.*[69]

late evening peak in B cell count is close to the time of maximal skin or bronchial reactivity to antigens such as house dust and/or penicillin in allergic patients.[2,38–40] B cells take part in this reaction.

The circulating count of T lymphocytes, as identified with a variety of techniques, doubled between morning (0800–1200 h) and early night (midnight–0400 h) (FIGURE 7).[10,12,13,21,35,36,41] A similar circadian pattern characterized the T helper subset [T (CD4+)].[2,36,41]

In mice, the count of circulating T lymphocytes also peaked in the early rest span.[42] A release of T lymphocytes at this circadian stage was suggested from:

(a) A circadian rhythm in circulating thymosin-1, a thymic hormone involved T cell maturation (and release?) which peaked in the early rest span in mice.[43]
(b) The appearance of presumably immature T cells in peripheral blood in the early night span in healthy subjects.[44]

Nocturnally released T lymphocytes may subsequently be distributed in peripheral tissues and cooperate with macrophages for improved efficiency at antigen recognition.

Potential pitfalls of such models of immune surveillance result from the scarcity of *human* data on circadian rhythms in T and B intraorgan proliferation, and on phagocyte activity, among other functions. The relationship of such a circadian immune clock with other oscillators will deserve further studies. For instance, the normal circadian rhythms in cortisol or testosterone secretions play a minor role if any in lymphocyte circulation,[41] but NK cells may exhibit a circadian-dependent susceptibility to cortisol.[45]

Circannual Time Structure of Immunity

Immune defenses are also organized along other time scales. These are reviewed elsewhere in detail.[36]

TABLE 1 indeed documents that winter depression of T cell immunity may characterize most living beings including man. In these studies, such circannual rhythms remained similar even when ambient temperature and photoperiod duration were kept constant throughout the year.[46–56] The circannual time structure of immunity is thus endogenous.

Furthermore, circannual rhythms may modulate the circadian time structure of some immune functions. Thus, the circadian rhythm in peripheral counts of T (CD3+) and T (CD4+) lymphocytes was not detected at a group level in March or in June but had a large amplitude in August, November, and January (FIGURE 8).[13] The lack of a circadian rhythm at a group level may result from interindividual desynchronization, for example, individual circadian rhythms may exhibit period lengths differing from precisely 24 h. This phenomenon might be more likely to occur at specific times of the year, as shown for testosterone and lutropin secretions and elsewhere discussed.[13,36]

The prominent group-synchronized circadian rhythm in the density of CD3 molecules on the surface of T lymphocytes (FIGURE 4) was observed in March, when the peripheral count of T (CD3+) lymphocytes lacked any group circadian rhythm. This indeed indicates that both systems may be "physiologically" desynchronized.

TABLE 1. Winter Depression of T Cell Immunity

Species	Environment		Author (Year)
	Light-Dark	Temperature	
Lizard	Natural	Natural	Hussein, 1979[70]
Rainbow trout	Natural	Constant	Yamaguchi, 1981[71]
Guinea pig	LD 12 : 12	22 ± 1°C	Godfrey, 1975[49] Hildeman, 1980
Ground squirrel	Natural	23 ± 1°C	Sidky (1972)[46]
Mice	LD 12 : 12	23 ± 1°C	Brock, 1983[48] Pati, 1988[50]
Beagle dog	Natural	Natural	Shifrine, 1980[53]
Man	Natural	Natural	

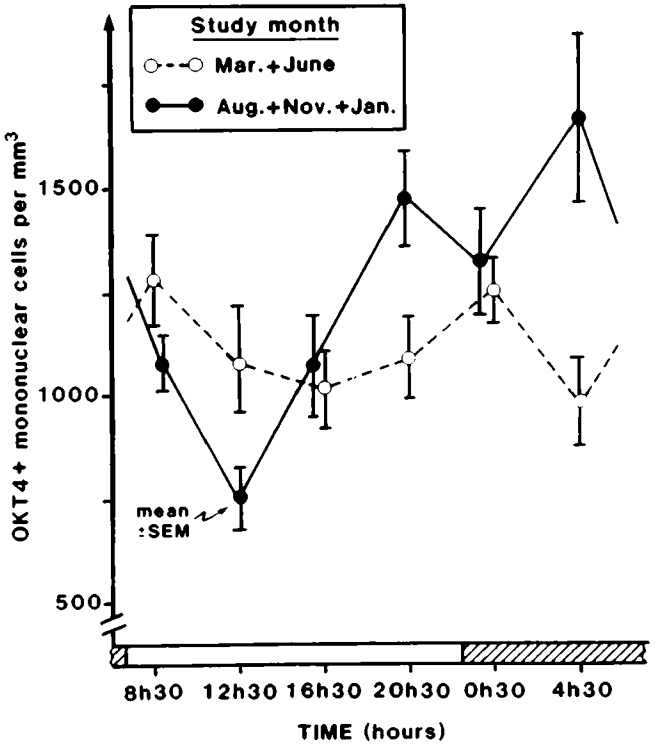

FIGURE 8. Seasonal modulation of the circadian time structure of circulating T helper lymphocytes T (CD4+) in five healthy young men. Circadian variation in January, August, and November, as opposed to March and June. Data are expressed as percentages of each individual's 24-h mean for each study month. Cosinor analysis yielded the respective following parameters (a) for March and June, $p = 0.42$ and $M = 1013 \pm 50$ cells/mm^3; (b) for August, November, and January, $p < 0.0001$, $M = 1230 \pm 50$ cells/mm^3, double amplitude = 52 ± 20% of mesor, peak time = 1.00 ± 1.20 h. After Lévi *et al.*[13]

Other Infradian Immune Rhythms

Other periodicities—circamenstrual (about 30 days) and circaseptan (about 7 days)—also modulate immune defenses. An extensive literature review led us to hypothetize that once an immune response has been triggered, its various cellular and humoral components become organized along multiples of 7 days as shown for the incidence of allograft rejection, both in laboratory rodents and in transplant patients.[57]

NK cell activity was shown to predictably double along the estrous cycle in female mice. Highest NK cell activity was highest near the middle of the estrous cycle (proestrus) in female mice. This rhythm appeared to influence the metastatic potential of a transplanted mammary tumor.[58] Similar results were reported in premenopausal women with breast cancer: the risk of recurrence was fourfold larger in women who had the tumor removed around menstruation as compared to ovulation.[59]

The knowledge of the time structure of immune defenses may be essential for understanding physiopathological processes. For instance experiments in mice have indicated the role of time of antigen exposure upon the generation of an allergic process.[60] The circadian coordination of immune functions also profoundly influences the effects of medications that either depress or stimulate immunity.

Chronotherapy with Biologic Response Modifiers

The dosing time of ciclosporin A (CiA) indeed influenced the extent of its immunosuppressive effect in mice.[61] Lentinan, an immunostimulant, was administered to rats prior to tumor inoculation: tumors grew faster or more slowly according to both time of administration and season of treatment.[62] Major differences in the toxicities of CiA[61,63] and tumor necrosis factor,[64] a cytokine used for treating cancer, were found according to dosing time in mice. Preclinical and early clinical chronopharmacologic studies have been performed with interferon (IFN) and will be reported.

IFN is a glycoprotein that is naturally produced by leukocytes. This agent has proved effective against metastatic renal cell carcinoma, among other malignancies. Its antitumor effectiveness was clearly dose related.[65] Toxicities of IFN may be severe and are dose limiting. They consist of fever, chills, malaise, obnubilation, asthenia as well as neutropenia, thrombocytopenia, and liver or renal damage. Various schedules and routes of administration have been used. This drug is usually injected intramuscularly 3 times per week at doses ranging from 3 to 9 MU/m2 per day. Doses of 3–5 MU/m2 per day were recommended for continuous venous infusion of this drug.[66] Evening intramuscular injection was found less toxic than morning ones in patients.[67] In a crossover study, IFN was injected either at 0800 h or at 2000 h to healthy subjects; plasma cortisol and circulating T and NK lymphocyte subsets were measured every 3–6 h for 24 h. Circadian rhythms in these variables were similar both in the absence of IFN injection and following drug administration at 2000 h. IFN dosing at 0800 h resulted in a profound alteration of these physiologic rhythms, which likely relate to the increased toxicity of morning IFN.[68]

Preclinical Findings

The ability of alpha IFN to stimulate NK cell activity was examined in mice at different circadian stages. Male B6D2F1 mice were randomly allocated to one of six groups of 10 animals. Each group was housed on a different shelf of an autonomous chronobiologic facility (ESI-Flufrance, Arcueil, France). Such a facility allows one to synchronize mice in six different lighting schedules consisting of an alternation of 12 h light (L) and 12 h dark (D). The phasing of circadian rhythms can be shifted by altering the light-dark cycle. Food and water were provided *ad libitum*. Light onset was staggered by 4 h 15 min in each shelf with temperature being automatically controlled (23–25°C). Light intensity ranged from 600 to 800 lux at 30 cm from the source. After 3 weeks of synchronization, the mice had adjusted to the altered lighting schedule, and six circadian stages could be explored at the same clock hour. Mice from each circadian group were sacrificed at 3, 6, 10, 13, 18, or 22 h after light onset (HALO). Spleens were removed. Three suspensions per time point (each corresponding to 3 or 4 spleens)

were prepared in RPMI 1640 culture medium by gently teasing apart the organs between the frosted ends of two microscope slides. After filtration through a nylon gauze, the suspensions were centrifuged at 300 × *g* for 7 min at 4°C. Cells were resuspended into medium and their viability was assessed by trypan blue dye exclusion test. NK cell activity of splenocytes was assessed from 51 Cr release by YAC-1 lymphoma cells, following 4 h exposure, as previously described.[50] A circadian rhythm characterized splenic NK cell activity with maximal value in the early activity span of mice, similar to that shown in FIGURE 4. *In vitro* exposure of splenocytes to mouse IFN (1000 U/ml for 1 h) resulted in an increase of NK cell activity which was fourfold greater in the second half of the active span of mice as compared to exposure in the late rest or early activity spans (FIGURE 9).

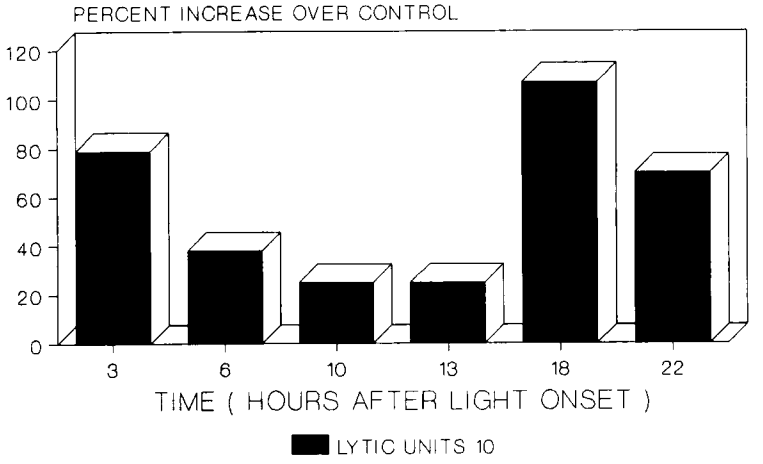

FIGURE 9. *In vitro* stimulation of murine splenocyte NK activity with alpha interferon (IFN) as a function of circadian time of exposure. Splenocytes sampled in the mid to late activity or early rest span of mice (18, 22, or 3 hours after light onset) and subsequently exposed to several concentrations of IFN exhibited greatest increase in NK cell activity.

Clinical Study

We hypothetized that alpha IFN would be more effective and less toxic in the late evening in man. A phase I clinical trial was undertaken in patients with metastatic carcinoma. IFN was continuously infused for 21-day courses every month (10 day's rest), at doses ranging from 15 MU/m2 per day according to an intrapatient escalation protocol. Drug delivery was automatically modulated along the 24-h scale from 0.002 ml/h between 0600 h and 1000 h up to 0.09 ml/h between 1800 h and 2200 h, using a small ambulatory pump (SP 404 pump, Minimed, France) (FIGURE 10). This device has a 3-ml reservoir which was connected to the central venous system via an implanted access port. Syringe changes were required every 3 days. IFN was concentrated up to 30 MU/ml.

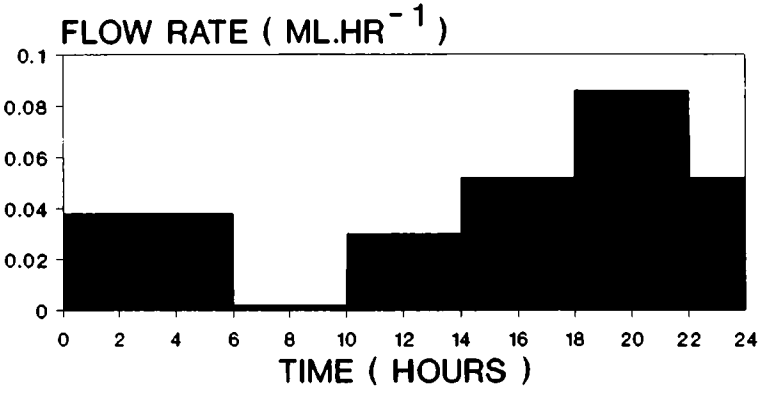

FIGURE 10. Schedule of venous delivery of alpha interferon over 24 h in patients with renal cell carcinoma. Automatized circadian changes in flow rate were performed with a small ambulatory pump (Minimed SP 404).

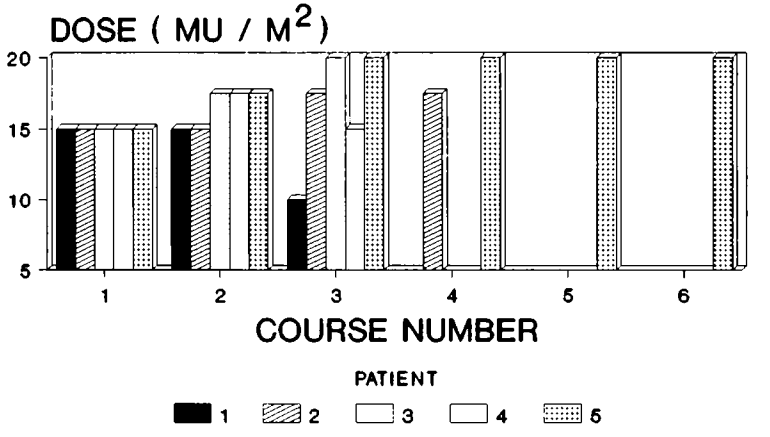

FIGURE 11. Continuous venous chronotherapy with alpha interferon. Tolerance of patients with metastatic renal cell carcinoma as gauged by daily doses given for 3–6 courses. Preliminary results of an ongoing phase I trial.

All patients tolerated the first dose level for the whole 21-day course. Five patients have received at least 3 courses at present. These very high doses were safely infused to four of them, for 3–6 months (FIGURE 11). All remained ambulatory and two of them continued their professional activities during infusional chronotherapy. A minor response was documented in three heavily pretreated patients. These preliminary results suggest that chronotherapy with BRMs may profoundly improve their therapeutic index, as a result of an increase in both dose intensity (circadian stage of least host toxicity) and desired pharmacodynamic effect (circadian stage of highest immune cell susceptibility). Programmable-in-time pumps are now available for such chronotherapy with high drug doses and minimal side effects to be performed in ambulatory patients.

SUMMARY

Immune defenses are organized along both 24-h and yearly time scales. Two circadian systems have been isolated in man, which can be desynchronized: (1) the circulation of T, B, or NK lymphocyte subsets in peripheral blood and (2) the density of epitope molecules (CD3, CD4, . . .) at their surface, which may relate to cell reactivity to antigen exposure. The *in vitro* response of murine splenocytes to interleukin 2, interferon (IFN), or cyclosporin A strongly depended upon circadian time of exposure. Temporally optimized delivery of biologic response modifiers (BRM) may be guided by immunologic marker rhythms. An alternative yet complementary strategy was sought with IFN: since high doses were shown as more effective than low doses against several malignancies, this drug was given at the presumed less toxic time, so that its dose could be increased. Continuous drug delivery was circadian modulated in 8 cancer patients. Dose intensities twice to fourfold higher than those usually recommended were safely infused to ambulatory patients. Chronotherapy with BRM may represent a necessary step for optimizing the immunologic control of malignancies.

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