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Seasonal variation in peripheral blood leukocyte subsets and in serum interleukin-6, and soluble interleukin-2 and -6 receptor concentrations in normal volunteers

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Abstract. This study has been carried out in order to investigate seasonal variation in peripheral blood immune cells, such as leukocytes, monocytes, neutrophils, lymphocytes, $CD3^+$ T, $CD4^+$ T, $CD8^+$ T, $CD25^+$ T, $CD20^+$ B, and serum interleukin-6 (IL-6), soluble IL-6 receptor (sIL-6R) and sIL-2R levels in normal volunteers. Toward this end, 26 normal volunteers (13 men, 13 women) had monthly blood samplings during one calendar year for peripheral blood count, flow cytometric enumeration of peripheral leukocyte subsets and immunoassays of IL-6, sIL-6R and sIL-2R. It was found that most of the immune variables change rhythmically during the seasons as a group phenomenon. Statistically significant yearly variations with seasonal rhythms, i.e. annual rhythms or harmonics, such as semiannual, tetramensual and trimensual rhythms, were found in the number of leukocytes, neutrophils, monocytes, lymphocytes, $CD4^+$ T, $CD8^+$ T, $CD25^+$ T, $CD20^+$ B cells, in the $CD4^+/CD8^+$ ratio, and serum IL-6 and sIL-6R levels. It is concluded that the immune system is characterized by a multifrequency time-structure with significant high-amplitude yearly variations in the number of some peripheral blood leukocyte subsets.
Key words. Interleukins; leukocytes; lymphocytes; seasons; annual rhythms; chronbiology.

The immune system is characterized by a multifrequency time structure with significant periodicities in cell function, proliferation and percentage or number of peripheral blood leukocytes subsets[1-3]. High amplitude circadian rhythms in number or function of leukocyte subsets are now well documented[1,2,4-6]. Comparatively fewer studies have focused on the occurrence of seasonal variation in function or counts or peripheral blood mononuclear cells (PBMC). Most[7-11] but not all[12] groups observed significant seasonal differences in total number of peripheral blood leukocytes with peaks occurring in winter (maxima around December–March) and troughs in summer (May–August). Seasonal variation in number of circulating T and B lymphocytes, as determined by means of bacterial adhesion tests, have been described in healthy volunteers: number of T cells peaked in late fall, whereas B cells peaked in winter[13]. Lévi et al.[14] found significant annual or semiannual rhythms in number of various leukocyte subsets, e.g. total lymphocytes, $CD3^+$ T, $CD4^+$ T, and $CD8^+$ T lymphocytes. Abo et al.[15] found significant circannual rhythms in the count of circulating B cells in Japan with peak values in summer and lows in winter, whereas MacMurray et al.[16] observed an opposite pattern in the USA. Circaseptan variations in number of circulating T and B cells, and in other immune-related functions are documented[3,7,17]. Seasonal variation in cell-mediated immunity is suggested by seasonal differences in lymphoproliferative responses to various lectins in man[18]. Circannual rhythms were also detected in lymphocyte blastogenic responses in mice: peaks occurred in March–June, with lows in December–January[19,20]. Canon and Lévi[21] concluded that winter depression of T cell immunity may characterize most living beings including man. Circannual variations have also been found in inflammatory responses, e.g. carageenan-induced paw edema in the rat peaks in spring, whereas lows are observed in winter[22].

A significant seasonal pattern of maximum development of immune-related disorders has been demonstrated. There is strong evidence of seasonal variations in the incidence of various infectious diseases[11,14]. A true seasonal variation has been observed in the diagnosis and symptomatology of and mortality due to breast cancer and in the occurrence of other neoplasias[6,14,23-25]. Finally, it has been demonstrated that there are time-dependent, seasonal changes in the therapeutic ratio of anticancer drugs[26].

However, the above research on seasonal variation in leukocyte subsets of normal volunteers was often limited by the cross-sectional design of the studies, the small number of healthy subjects investigated (e.g.,

n = 5, n = 9) in longitudinal studies, the small number of time-points for blood sampling (e.g., 2–5 time-points in one calendar year), the measurements of leukocyte subsets (e.g., bacterial adhesion tests or limited number of leukocyte subsets measured), and by the statistical analyses employed (e.g., analyses of variance). Moreover, no previous study has examined possible seasonal variations in serum levels of interleukins or their receptors.

The present prospective study was carried out in order to examine the yearly variation in number of peripheral blood leukocytes, lymphocytes, monocytes, neutrophils, $CD3^+$ T, $CD4^+$ T, $CD8^+$ T, $CD25^+$ T and $CD20^+$ B lymphocytes and in serum interleukin-6 (IL-6), soluble IL-6 receptor (sIL-6R) and sIL-2R concentrations. Therefore, a larger number of normal volunteers had monthly blood samplings during one calendar year for flow cytometric or ELISA assays of the above variables. The data were analyzed by means of least squares cosine spectral analysis in conjunction with cosnior fit of single time series or a group of time series and multiple regression analysis[27–29].

Subjects

The geographical coordinates for this study were 51.2 °N and 4.5 °E around the city of Antwerp, Belgium. Normal Caucasian volunteers were selected to participate in this study. Inclusion criteria were: a) the fact of living in Antwerp, in an area <30 km from the Meterological Station, Royal Meterological Institute, Deurne, Antwerp, Belgium; b) a stable, settled life-style; and c) physical and psychological health. All volunteers were free of major medical illnesses (e.g., immune or endocrine illness). All volunteers were free of any drugs (including the pill). They were screened for past history of psychiatric and personality disorders by means of the Semi-Structural Clinical Interview of the DSM-III-R[30,31]. None of the subjects had suffered from psychiatric disorders or personality disorders. All had scores <40 on the Zung Anxiety and Depression Inventories, and <9 on the Beck Depression Inventory, thus excluding subjects with psychological stress or anxiety. Subjects were not allowed to spend more than one week in a geographical area other than the province of Antwerp; they were not allowed to travel outside an area of more than 250 km from Antwerp-town. Subjects regularly taking international flights were not included. Subjects who travel a lot, such as commercial travellers, were not included in this study. Subjects with known drug use or abuse (alcohol, and any other drugs of dependence) were not included. In addition, women of childbearing potency were only included if they were willing to avoid pregnancy during this study span.

Exclusion criteria were: a) abnormal chemical and hematological tests in the first study month. The tests included complete blood count, blood urea, nitrogen, electrolytes, liver enzymes (SGPT, SGOT, γGT), hemoglobin, hematocrit, and thyroid function; b) presence of acquired immunodeficiency syndrome; c) subjects with premenstrual tension syndrome; d) occurrence of medical illnesses during the study span, except a common cold or angina; e) subjects starting with any medical drugs, except occasional use of an over-the-counter drug such as aspirin; f) the occurrence of important negative events in life during the study span; and g) tobacco use of more than five cigarettes a day. In case of a common cold or angina, blood samplings were deferred until clinical remission. Use of over-the-counter drugs, such as aspirin, was prohibited for at least two days prior to blood sampling. After a vacation in an area other than the province of Antwerp, blood was sampled at least seven days after returning to Antwerp. Blood samplings in premenopausal females were carried out 5–10 days after the first day of the menstrual cycle. Finally, 26 normal controls (13 men, 13 women; mean age = 38.7 ± 13.4 years; range: 23–69 years) were selected to participate in this study. They gave oral informed consent to participate in the study in accordance with the ethical standards of the Ethical Committee of the University of Antwerp (UIA), Belgium. All controls had the socio-economical status of the middle-class Belgian population, and a mean, net monthly income between $1,500 and $2,500. Subjects were all urbanized persons with a comparable rest–activity schedule. The study period extended from December 11, 1991 until December 25, 1992. Sixteen subjects had their first blood sampling in December 1991, the others started in January 1992. Seasons were defined by their respective solstices and equinoxes, i.e. winter: December 21–March 20; spring: March 21–June 20; summer: June 21–September 20; and fall: September 21–December 20. Cross-seasons were defined as the periods between: 1) November 6–February 5; 2) February 6–May 5; 3) May 6–August 5; and 4) August 6–November 5.

Methods

The preparation of the subjects prior to blood collection was carefully controlled and the collection itself was performed in standardized conditions in order to minimize sources of preanalytical variation. Blood was sampled after an overnight fast at 08.00 h. The same two scientists carried out all blood samplings throughout the study span. Each of them was allocated to one of the subjects, so that the latter always had blood sampled by the same investigator. Subjects had 12 consecutive monthly blood samplings during the study span. Blood samplings were clustered in ±5 blood sampling days/month. During the study span, there were 59 days on which blood was sampled.

An intravenous cannula was inserted at 08.00 h in the antecubital vein of the subjects. Sixty-five mL of blood

was taken during a period of 30 min. Blood sampling procedures were kept constant throughout the study span. Serum for the determination of IL-6, sIL-6R, and sIL-2R was stored at −70 °C until thawed for assays. All blood samples of one and the same subject were always assayed in the same run. IL-6 was quantified with a sandwich ELISA (Eurogenetics, Tessenderlo, Belgium) based on a monoclonal–monoclonal antibody pair and a biotin–streptavidin amplification system. The dynamic range of the immunoassay varies between 0 and 500 pg/mL; the intra-assay coefficient of variation (CV) is 3.9% at 10 pg/mL. Standardization of sIL-2R measured by the sIL-2R ELISA (Eurogenetics) kit is expressed in arbitrary units and ranges between 20 and 1600 U/mL. Each unit corresponds to approximately 3.0 pg/mL pure recombinant α-chain receptor. The intra-assay CV value is 2% at a concentration of 382 U/mL. The assay of sIL-6Rs was performed with the sIL-6R ELISA (Eurogenetics), which is a monoclonal antibody-based sandwich ELISA standardized on a 20 to 800 ng/ml calibrator range. The intra-assay CV values vary between 4.0% and 7.8% at the corresponding serum concentrations of 50 ng/mL and 500 ng/mL, respectively. Flow cytometry was always performed on fresh blood. EDTA-blood (0.1 mL) was mixed with 20 µL of a fluorescein-labelled monoclonal antibody and 20 µL of a phycoerythrin-labelled antibody. After incubation of 15 min at 4 °C, red blood cells were lysed with NH_4CL solution for 20 min at room temperature. The cells were washed and fixed in phosphate-buffered saline (PBS) with 1% paraformaldehyde. Afterwards, the lymphocytes were analyzed on a FACSCAN flowcytometer using SIMULSET software (Beckton Dickinson, Mountain View, CA, USA). Labelled monoclonal antibodies against CD4, CD8, CD3 and CD25 were purchased from Dakopatts (Copenhagen, Denmark) and CD20 from Becton Dickinson. The following antibodies were used: $CD3^+$ (pan T cells), $CD4^+$ (T 'helper' cells), $CD8^+$ (T 'suppressor/cytotoxic' cells), $CD25^+$ (interleukin-2-receptor bearing T lymphocytes) and $CD20^+$ (B lymphocytes). All measurements of the different leukocyte subsets in the present study were always obtained using a same lot of monoclonal antibodies. Quality control performed through calibration with Quick Call (Becton Dickinson) showed that highly reproducible results were obtained in our laboratory during the course of this study. White blood cell count and leukocyte differentiation (lymphocytes) were performed on a Technicon HI fully automated total blood cell counter. To obtain the white blood cell differential, three parameters of the sample are simultaneously measured in the flow cell, i.e. volume, radiofrequency conduction and light scatter. The interassay CV values were for leukocytes: 1.29% and for lymphocytes: 2.46%. Absolute counts for the lymphocyte subsets were derived using this white blood cell count, the lymphocyte fraction and the proportion of antibody positivity from the FACS analysis. Thus, the results were expressed as the percentage of positive lymphocytes and as the absolute number of cells bearing the surface markers. For reasons of clarity, however, this paper reports only the number and not percentage of PBMC. Complete lists with results on percentage of PBMC can be requested from the authors. The technical and administrative aspects of all laboratories involved in this research remained constant during the study span.

Statistics

Repeated measures analyses of variance (ANOVAs) were used to investigate various sources of variance, i.e. interindividual variability with gender and age (<35 years versus ≥ 35 years) effects, intraindividual variability with seasonal, cross-seasonal or monthly differences, and two- or three-way interactions between time × age, time × sex, and time × sex × age. Multiple comparisons among treatment means (e.g., cross-seasons) were checked by means of the Dunn test. Relationships between variables have been assessed by means of Pearson's product moment correlation coefficients, pooled over the time series of the 26 normal volunteers (in order to eliminate interindividual variability).

Rhythmometry in time series was ascertained by means of least squares spectral analysis of a single time series or a group of time series and by means of multiple regression and cosinor fit analysis[27]. For a comprehensive review of the theoretical backgrounds of the procedures employed in the present study, the reader is referred to previous publications of one of the authors[27]. The statistical methods described by De Meyer and Vogelaere[27] are, in part, based on those of other authors[32–35]. Spectral analysis searches for hidden periodicities in a single time series or in a group of time series on a probabilistic basis and allows an increased scanning of the whole frequency range. The significant rhythms are identified by relatively sharp peaks rising above a continuous background. The only guarantee that these peaks correspond to real physical mechanisms is a significant signal-to-noise ratio in the spectral peaks. F-statistics are generated as a measure of the signal-to-noise ratio for each of the rhythms investigated and are listed in a periodogram or F-spectrum. In the present study, up to 100 frequencies (rhythms) are scanned in a range between 2 and 366 days. Group spectral analysis were carried out on the time series of the immune variables in the 26 subjects; the within + between F-spectra were interpreted to make inferences on common rhythms expressed at the population level. Spectral analysis of single time series were performed on the pooled time series of the healthy volunteers after normalization of the immune data relative to the yearly mean of the monthly measurements in each of

the subjects. This normalization eliminates the inter-individual variability in the data. The total amount of variance in the immune data, explained by the various significant rhythms (identified by means of the peak F-values in the F-spectra) is computed by means of multiple regression analysis, i.e. time series of immune data as dependent variable and the various significant rhythms as explanatory variables. The predicted values are used as an index of the cyclic signal in the time series[28,29]. These values are, subsequently, extrapolated to all the days in the study period in order to display the cyclic signal subtracted from the raw data. This procedure allows to delineate a multiple component model with estimates of orthophase and range from low to peak values in a time series with different rhythmic components, as well as the relative contribution of each of the significant rhythms in explaining the variance in the raw data. The results of these multiple regression analyses are checked for autocorrelation by means of the Durbin-Watson statistic[36].

Results

Table 1 lists the results of repeated measures ANOVAs, which considered the cross-seasons as repeated measures, and sex and age as factors (complete lists with the results of repeated measures ANOVAs with months or seasons as treatments can be requested from the authors). Most immune variables show significant differences between the cross-seasons, $CD3^+$ T and sIL-2R being the exceptions. There were no significant age × sex × time, age × time, or sex × time interactions for any of the variables investigated. Statistically highly significant monthly differences were found in number of monocytes ($F = 5.0$, $df = 11/233$, $p < 10^{-4}$). There were significant monthly differences in $CD3^+$ T cells ($F = 2.2$, $df = 11/230$, $p = 0.02$). No monthly (or seasonal) differences could be detected in sIL-2R.

Table 2 shows the outcome of group spectral analyses performed on the time series of the immune variables in 26 normal volunteers; listed are the frequencies (in days) of the peak F-values (and the range in days by which significant F-values were observed). Significant ($p < 0.05$) circannual rhythms were observed in lymphocytes, $CD4^+$ T, $CD8^+$ T, $CD20^+$ B cells, and $CD4^+/CD8^+$ ratio; semiannual rhythms in neutrophils and $CD25^+$ T cells; and trimensual or tetramensual rhythms in $CD4^+$, $CD8^+$ and $CD25^+$ T cells.

Cosinor fit of annual, semiannual, and trimensual rhythms on the pooled time series of the normalized values in each subject, showed significant annual rhythms in lymphocytes ($p = 0.02$), $CD4^+$ T ($p = 0.0002$), $CD8^+$ T ($p = 0.0002$), $CD20^+$ B ($p < 10^{-4}$), and $CD25^+$ T ($p = 0.004$) cells, $CD4^+/CD8^+$ ratio ($p < 10^{-4}$), and IL-6 ($p = 0.01$); significant semiannual rhythms in absolute number of neutrophils ($p =$

Table 1. Cross-seasonal differences in immune-inflammatory variables in 26 normal controls.

Mean values (±SD)					ANOVAs*			Dunn test		
Variables	Nov. 6–Feb. 5 (1)	Feb. 6–May 5 (2)	May 6–Aug. 5 (3)	Aug. 6–Nov. 5 (4)	F-statistic	df	p-value	Contrasts	T statistic	p-value
Leukocytes	6.2(2.8)	6.6(3.2)	5.9(1.9)	6.3(2.4)	3.0	3/263	0.03	(3) vs (1, 2, 4)	2.6	0.01
Neutrophils	3.6(1.8)	4.5(2.8)	3.5(1.5)	3.8(1.9)	8.9	3/241	$<10^{-4}$	(2) vs (1, 3, 4)	4.4	$<10^{-4}$
Lymphocytes	2.10(0.83)	1.74(0.62)	1.96(0.67)	2.02(0.73)	7.9	3/241	0.0001	(2) vs (1, 3, 4)	4.7	$<10^{-4}$
Monocytes	0.43(0.16)	0.48(0.20)	0.43(0.14)	0.42(0.18)	2.8	3/241	0.04	(2) vs (1, 3, 4)	2.8	0.006
$CD3^+$	1.67(0.71)	1.60(0.70)	1.55(0.56)	1.57(0.61)	1.3	3/238	0.3	-	-	-
$CD4^+$	1.34(0.61)	1.22(0.55)	1.11(0.44)	1.22(0.53)	8.3	3/239	0.0001	(1) vs (2, 3, 4)	4.5	$<10^{-4}$
	-	-	-	-	-	-	-	(3) vs (2, 4)	3.2	0.002
$CD8^+$	0.47(0.20)	0.55(0.23)	0.53(0.19)	0.46(0.18)	4.9	3/239	0.003	(2, 3) vs (1, 4)	3.6	0.0006
$CD4^+/CD8^+$ ratio	3.2(2.0)	2.4(1.1)	2.2(0.9)	3.0(1.8)	11.3	3/256	$<10^{-4}$	(2, 3) vs (1, 4)	5.8	$<10^{-4}$
$CD20^+$	0.37(0.16)	0.27(0.11)	0.26(0.10)	0.30(0.12)	17.0	3/237	$<10^{-4}$	(1) vs (2, 3, 4)	6.9	$<10^{-4}$
$CD25^+$	0.090(0.055)	0.099(0.077)	0.070(0.076)	0.138(0.157)	12.0	3/239	$<10^{-4}$	(4) vs (1, 2, 3)	4.1	0.0002
	-	-	-	-	-	-	-	(3) vs (1, 2)	4.4	$<10^{-4}$
IL-6 (pg/mL)	7.2(4.6)	6.7(3.7)	6.9(4.4)	5.6(4.2)	4.4	3/260	0.005	(4) vs (1, 2, 3)	3.5	0.0008
SIL-6R (U/mL)	162(62)	168(69)	164(62)	174(70)	4.9	3/275	0.003	(4) vs (1, 2, 3)	2.9	0.004
SIL-2R (U/mL)	410(112)	402(94)	413(103)	408(105)	0.6	3/275	0.6	-	-	-

All results are expressed as absolute number, in cells 10 E9/L of leukocytes or leukocyte subsets. All results of repeated measures ANOVAs with cross-seasons as repeated measures, sex and age groups (35 years vs ≥35 years) as factors.

Table 2. Results of group spectral analysis on the time series of immune variables in 26 normal controls.

Immune variables	Significant rhythms (days) subtracted from the between + within F-spectra*
Neutrophils	169 (148–191)
Lymphocytes	344 (293–366)
Monocytes	50 (49–51), 78 (75–79)
$CD4^+$	35, 82 (75–86), 49, 24, 64, 366 (278–366)
$CD8^+$	97 (86–111), 330 (268–366), 46, 24, 35
$CD4^+/CD8^+$ ratio	351 (235–366), 46 (46–49), 100 (89–108), 24, 35
$CD20^+$	366 (257–366), 111 (93–122), 24 (17–24), 35, 60 (53–60)
$CD25^+$	209 (129–297), 93 (74–118), 24 (20–27)

The immune variables were analyzed as the absolute number of cells.
*Listed are the significant rhythms (in days) subtracted from the between + within F-spectra generated by means of group spectral analyses on the 26 time series of the 26 normal controls (significance level <0.05). Listed are the rhythms, which show a peak value in the F-spectra and (between parentheses) the range of significant rhythms.

0.00004), $CD25^+$ T ($p = 0.0001$), and sIL-6R ($p = 0.02$); significant trimensual rhythms in $CD3^+$ ($p = 0.03$), $CD8^+$ ($p = 0.00003$), and $CD25^+$ ($p < 10^{-4}$) T cells and $CD4^+/CD8^+$ ratio ($p = 0.003$).

Table 3 shows the outcome of 12 multiple regression analyses, with the immune variables as dependent variable and the significant rhythms (peak F-values as determined by spectral analysis on the pooled time series) as explanatory variables. An important part ($>10\%$) of the variance in monocytes, $CD4^+$, $CD8^+$, $CD25^+$ T, and $CD20^+$ B cells and in the $CD4^+/CD8^+$ ratio could be explained by various seasonal (i.e. annual or harmonics, such as semiannual, tetramensual or trimensual) or higher frequency rhythms. A smaller part ($<10\%$) of the variance in leukocytes, $CD3^+$ T, IL-6 and sIL-6R levels was explained by one or more (seasonal) rhythms.

Figure 1 shows the cyclic signal in number of neutrophils, lymphocytes and monocytes. There was a significant time-relationship between the time series of neutrophils and lymphocytes (pooled $r = -0.25$, $p = 0.0001$, $n = 269$) or monocytes (pooled $r = 0.26$, $p = 0.00008$, $n = 269$). Figure 2 shows the yearly variation in number of $CD4^+$ and $CD8^+$ T cells and the $CD4^+/CD8^+$ ratio. The latter shows a marked yearly variation with peaks in October–November and December–January–February, and troughs in June. There were significant time-relationships between the number of $CD4^+$ and $CD8^+$ T cells (pooled $r = 0.21$, $p = 0.0009$, $n = 267$). Figure 3 shows the yearly variation in number of $CD20^+$ B and $CD25^+$ T cells. There were significant time-relationships between number of $CD20^+$ B and $CD4^+$ T cells (pooled $r = 0.48$, $p < 10^{-4}$, $n = 265$) and the $CD4^+/CD8^+$ ratio ($r = 0.26$, $p = 0.00009$, $n = 265$). The amplitudes (i.e. the range of change from nadir to peak values observed in the cyclic signal and expressed as a pecentage of the mean) of the yearly variations in the above variables were as follows; neutrophils: 19.3%; lymphocytes: 20.4%; monocytes: 54.3%; $CD4^+$ T: 34.1%; $CD8^+$ T: 53.6%; $CD20^+$ T: 68.4%; $CD25^+$ T: 122%; and $CD4^+/CD8^+$ ratio: 96.2%. It is well known that high amplitude seasonal variations, such as observed in the above immune variables, may have diagnostic implications in that different population-based reference ranges could apply for different periods of the year (the so-called time-qualified refer-

Table 3. Results of multiple regression analyses with immune data as dependent variables and the significant rhythms (subtracted by means of spectral analysis) as independent variables.

Dependent variable	Significant rhythms (in days) with exact p-value of regression coefficients	Multiple regression analyses			
		R^2 (%)	F-statistic	df	p-value
Leukocytes	151 (0.003), 49 (0.03)	4.2	4.8	4/287	0.001
Neutrophils	158 (0.0002), 311 (0.02)	9.9	7.3	4/265	0.00007
Lymphocytes	347 (0.0003), 191 (0.02)	8.8	6.4	4/265	0.0002
Monocytes	53 (0.00003), 366 (0.02), 78 (0.06)	18.8	10.2	6/263	$<10^{-4}$
$CD3^+$	104 (0.01)	3.1	4.3	2/264	0.01
$CD4^+$	49 (0.001), 74 (0.02), 366 (0.001)	16.5	8.6	6/261	$<10^{-4}$
$CD8^+$	100 (0.0001), 355 (0.006), 46 (0.02)	20.2	11.0	6/261	$<10^{-4}$
$CD4^+/CD8^+$Ratio	366 ($<10^{-4}$), 97 (0.0002), 38 (0.006), 20 (0.01)	23.1	10.4	8/276	$<10^{-4}$
$CD20^+$	366 (0.00002), 60 (0.0002), 111 (0.0005)	25.4	14.7	6/259	$<10^{-4}$
$CD25^+$	93 ($<10^{-4}$), 366 (0.0002), 38 (0.0003), 183 (0.0005), 20 (0.0009), 202 (0.003), 27 (0.009)	41.5	12.8	14/253	$<10^{-4}$
IL-6	366 (0.008), 9 (0.07)	4.1	3.0	4/284	0.01
SIL-6 R	115 (0.0009), 206 (0.02)	8.1	6.6	4/298	0.0001

This table lists the results of 12 multiple regression analyses with the pooled time series of the immune variables as dependent variables and the significant rhythms (in days), as determined by means of spectral analysis, as explanatory variables.

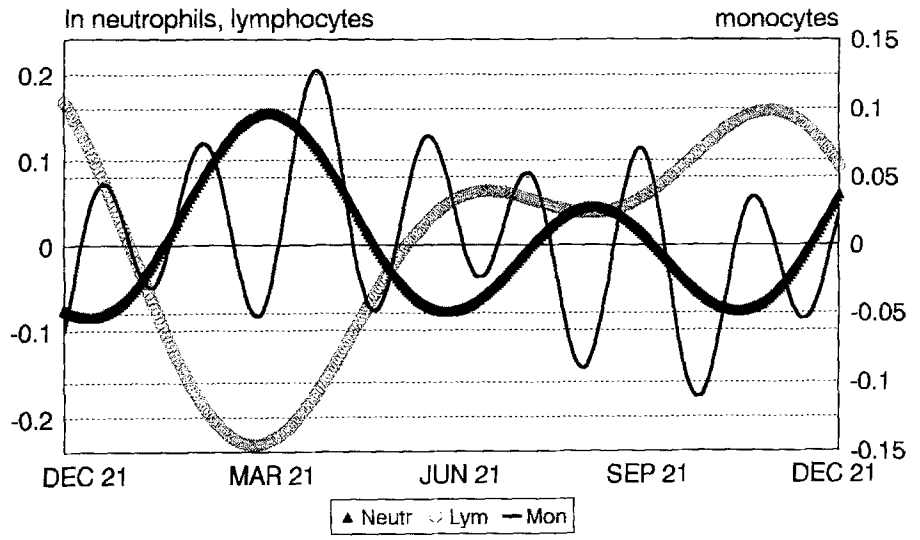

Figure 1. Yearly variation in absolute number of neutrophils (in ln transformation), monocytes and lymphocytes. This chronogram shows the cyclic signal in these immune cells which was subtracted by means of spectral analysis on the pooled time series of the normalized data (i.e. normalized relative to the yearly mean values in each subject).

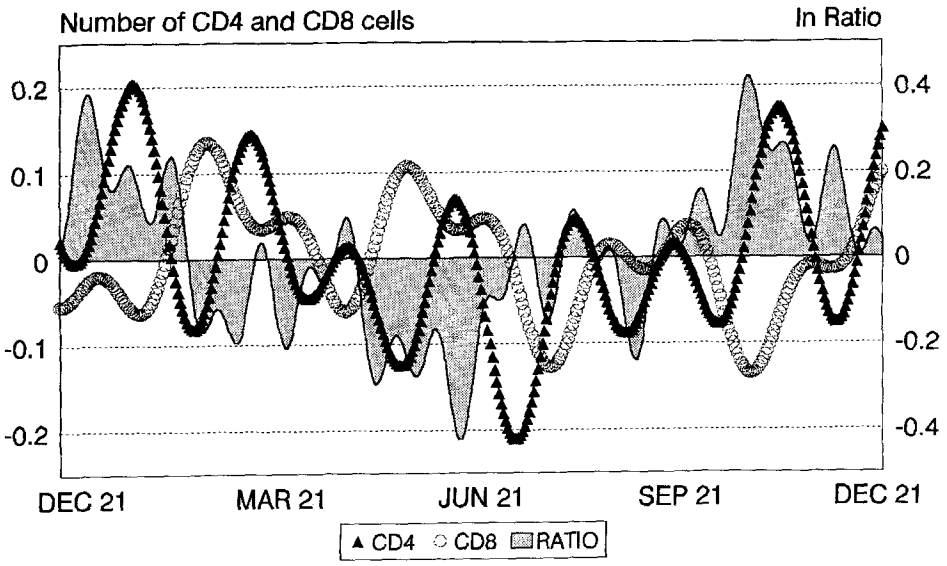

Figure 2. Seasonal variation in number of $CD4^+$ and $CD8^+$ T cells and the $CD4^+/CD8^+$ ratio (in ln transformation); results of spectral analysis on the pooled time series of the normalized data with reference to the yearly mean values in each subject.

ence ranges[37]. In order to check the utility of time-qualified reference ranges, the authors have computed the relative contribution of the total interindividual (i.e. interindividual + analytical variability) and total intraindividual (i.e. intraindividual + analytical variablity) variability to the total sum of squares of the immune variables. The interindividual variability was greater than the intraindividual variability for total number of WBC (i.e. 80.5% vs 19.5%), neutrophils (70.9% vs 29.0%), lymphocytes (67.3% vs 32.7%), $CD3^+$ (78.5% vs 21.5%), and $CD4^+$ (79.9% vs 20.1%) T cells. Monocytes (42.5% vs 55.0%), $CD8^+$ (39.2% vs 60.7%), $CD20^+$ (46.2% vs 53.6%), $CD25^+$ (12.9% vs 87.1%) T cells, and $CD4^+/CD8^+$ ratio (38.2% vs 61.8%) showed a lower interindividual than intraindividual variability. Consequently, population-based reference ranges are only useful for the number of monocytes, $CD8^+$, $CD20^+$, $CD25^+$ T cells, and $CD4^+/CD8^+$ ratio[38–41]. The percentage of the variability explained by monthly or seasonal differences was, for most variables, very low compared to the interindividual variability. For example, the percentage of the total sum of squares of the ln $CD4^+/CD8^+$ ratio attributable to monthly differences was 12.6% (compared with 61.8% and 38.2% for the intraindividual and interindividual components). Thus, the large inter- and non-seasonal intraindividual variablity in the immune data makes time-qualified ranges very broad in so far that the latter do not add much diagnostic value[41].

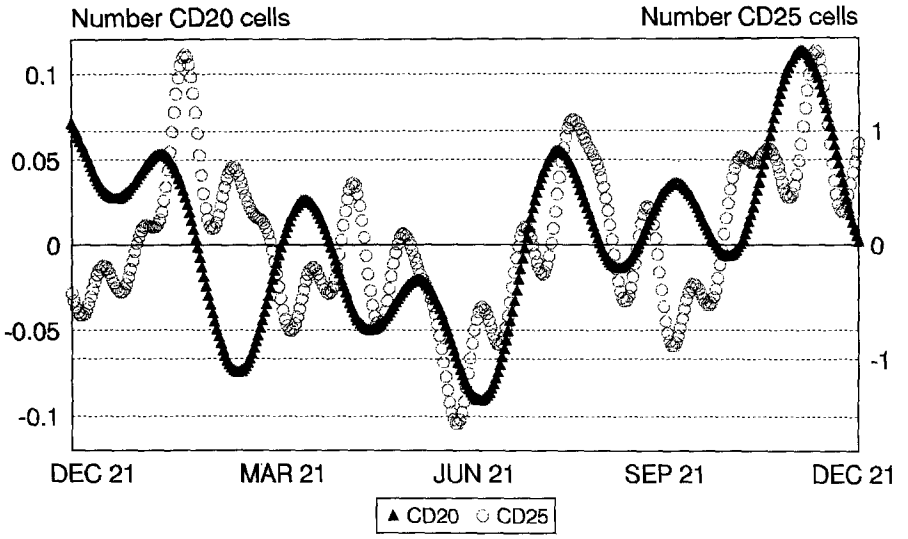

Figure 3. Seasonal variation in number of $CD25^+$ T (in ln transformation) and $CD20^+$ B cells; results of spectral analysis on the pooled time series of the normalized data with reference to the yearly mean values in each subject.

Discussion

The major finding of this study is that various leukocyte and lymphocyte subsets exhibit a high-amplitude yearly variation and change rhythmically as a group phenomenon, whereas the total number of leukocytes is relatively stable across the seasons. Neutrophils, lymphocytes, $CD3^+$ T, $CD4^+$ T, $CD8^+$ T, $CD25^+$ T and $CD20^+$ B cells, and $CD4^+/CD8^+$ ratio, show multiple, seasonal (i.e. annual and harmonics, such as semiannual, trimensual and tetramensual) rhythms expressed as a group phenomenon. It may be concluded that peripheral blood leucocyte subsets such as neutrophils and lymphocytes, and T and B lymphocyte subsets, show a rhythmical redistribution across the seasons. To our knowledge, this is a first report on seasonal variation in serum sIL-6R and IL-6 concentrations. However, the significant periodicities in these variables explained only a small part (± 4–8%) of the variance, while sIL-2R did not exhibit any seasonal variation.

The results of the present study are in part in accordance with previous studies reporting a seasonal variation with circannual or semiannular rhythms in lymphocytes, T cell subsets or B cells[14,15]. The discrepant results in peak and nadir occurrence of total leukocyte numbers, total lymphocytes and $CD4^+$, $CD8^+$, and B cells and the $CD4^+/CD8^+$ ratio that have been reported in the literature[10,13–15], may be explained by a number of factors. First, previous reports have often examined a lower number of subjects in a prospective study, performed cross-sectional studies or used a statistical approach that was less than optimal to detect the actual yearly variation in the immune data. Second, differences in geographical coordinates of the studies may account for some of the differences. Indeed, it is conceivable that the specific genetic constellation of our Flemish population (fairly homogeneous and stable for many centuries) and environmental factors (e.g. ambient temperature, light-dark cycle) may produce a specific seasonal variation in immune cells in this part of Belgium, if the significant immune rhythms are determined or modulated by endogenous (i.e. genetic) and/or environmental factors. Third, seasonal modulation or circadian rhythms may account for apparently discrepant results in peak occurrence of the rhythms. Indeed, there are a few reports that the circadian rhythms in total lymphocytes, $CD4^+$ and $CD4^+/CD8^+$ ratio may be different between some periods of the year[14]. Thus, the rhythms observed in PBMC could be due to: 1) alterations in mean 24 h number or percentage of PBMC; or 2) a seasonal modulation of the circadian time structure in the immune variables (with phase shifts or altered mesors). It appears from our study, that additional research on seasonal modulation of putative circadian rhythms in the number of PBMC may be limited to certain periods of the year, e.g., March, June, September and November, i.e. the periods with maximal changes in the levels of PBMC.

It is unlikely that the seasonal variation observed in immune cells is related to possible intervening effects of subclinical infections which may have occured in some subjects at some point during the study span (all subjects were free of clinical medical illness during the study span). It is obvious that the occurrence of subclinical infections in some subjects cannot explain the occurrence of seasonal rhythms as a group phenomenon. Rather, subclinical infections will induce a greater variability (intra- and interindividual) in the data, thereby reducing the significance of the actual seasonal variation.

Until now, there is limited information on the origin of the seasonal rhythms in the immune system. Two hy-

pothesis are: 1) the seasonal rhythms are endogenously (i.e. genetically) determined; 2) or are entrained by 'zeitgebers', such as the light-dark cycle or other climatic influences[21,42]. The first hypothesis is corroborated by the findings that, in experimental animals, circannual rhythms in T cell immunity occur independently from climatic factors and that there is a genetic difference in acrophase of antibody production between different strains of mice[21,43]. The second hypothesis is supported by the findings that changes in splenic weight, lymphocyte and monocyte counts may be induced by changes in temperature or photoperiod[44–46]. Neuroendocrine peptides and hormones, such as melatonin and cortisol, are candidate messenger-molecules transducing environmental signals to the body and the immune system[21,47]. Indeed, both hormones show a seasonal variation in man[48,49] and are known to have immune-modulatory activity[42,50]. Melatonin, for example, is an important transducer of photoperiodic information to endogenous rhythms, it is immune-stimulatory and antagonizes the immunosuppressive effects of glucocorticoids[21].

It is tempting to speculate that a relationship exists between the seasonal variation in leukocyte subsets observed here, and the increased incidence of immune-related disorders in some periods of the year (q.v. introduction). Thus, changes in $CD4^+$ T, $CD8^+$ T, and $CD20^+$ B cell number in winter versus fall and summer could alter the susceptibility/resistance to immune disorders. Phrased differently, changes in susceptibility/resistance rhythms in immune function across the seasons could maybe explain an increased incidence of infectious disorders in spring or in late winter[2,14,20,51–53] or of some cancers or neoplasias in spring–summer[14,23–25]. Future chrono-epidemiologic research could examine whether there is a relationship between the seasonal variations in leukocyte subsets observed here and immune-related disorders.

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