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Month-related variability in immunological test results; implications for immunological follow-up studies

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SUMMARY

This longitudinal study was originally designed to detect changes in the *in vitro* immune response of healthy subjects as a result of a psychological intervention In this study a significant proportion about 70%, of the immunological variability in the test results was accounted for by the differences in immunological response levels of the subjects Apart from this between-subject-effect, a significant proportion of the variability in test results was related to the month of data sampling The month effect was computed in such a way that the between subject variation was taken into account This resulted in a more accurate estimation of the month-effect Even after correction for the intervention, i e the defence of the PhD thesis the effect of month of data sampling remains significant for mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, percentage of CD4 and CD8 cells, and for the response to the mitogens phytohaemagglutinin, pokeweed mitogen and concanavalin A as well as the results for the mixed lymphocyte culture for one pool out of three In contrast, no significant month-effect was observed for the whole blood cell counts, for the differential white blood cell counts as determined by monoclonal antibody staining for cell surface markers CD3, CD16 TAC and OKM1, nor for the immunoglobulin IgM and IgG serum levels Likewise the cell-mediated lympholysis activities measured against three pools of stimulator cells remained unaltered We discuss the implications for future immunological follow-up studies of the observation that a significant proportion of the variability in immunological test results is related to differences between subjects and to the month of data sampling

Keywords circannual variability immunology follow-up studies

INTRODUCTION

The within-subject variability of test results over time is of principal interest for the monitoring of *in vitro* immunological parameters in subjects before and after clinical or other interventions Differentiating between experimentally-induced variation and other sources of variability is essential for the interpretation of the results Apart from between-subject variability, variation in immunological test results has been attributed to technical aspects diurnal variation and seasonal variation Test variation as the result of the use of different laboratory supplies during the period in which the study is done is widely recognized and easily controlled (Kiecolt-Glaser 1988) Another source of variation is due to the 24 h rhythm of some immunological activities (Tavadia *et al* 1975 Kaplan *et al* 1976) Therefore, most studies minimize the diurnal variation by keeping the hour of blood sampling quite constant (Kiecolt-Glaser 1988) A third source of variation, and less easily controlled is often described as being due to circannual or seasonal rhythms Although there is some evidence for a month of-data-sampling-effect on different immunological parameters the results presented by different authors (Reinberg *et al* 1977, Rocker *et al*, 1980, Bratescu & Teodorescu, 1981, MacMurray *et al*, 1983, Munch-Petersen, Wallevik & Faber 1985, Williams *et al*, 1986, Melnikov *et al*, 1987, Boctor, Charmy & Cooper 1989) are equivocal The number of immunological parameters studied is often small as is the number of subjects Furthermore the centres where these studies are carried out differ in their climate hours of light and temperature Since the results seem to vary with climatological conditions, i e over years and over geographical areas they cannot be used to compute a generally applicable correction factor for the time-effect Therefore it is necessary to incorporate the correction for month-specific effects into the statistical analysis of the immunological data This statistical method is described in this study

Our longitudinal study was originally designed to detect changes in the *in vitro* immune response in relation to the

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Table 1. Number of observations per month

Month	I	II
1	2	0
2	17	7
3	21	11
4	15	7
5	18	11
6	26	16
7	18	10
8	12	3
9	25	14
10	15	8
11	21	9
12	24	14
Missing	21	5
Total	235	115

I, Number of observations for all immunological variables except for cell mediated lympholysis (CML) II number of observations for CML

psychological stress of the defence of a PhD thesis In the course of analysing the data, we noted a strong effect on some but not all *in vitro* immunological measures tested It appeared that this effect could be completely explained by the month in which the blood samples were gathered Furthermore, for each immunological variable a significant subject-effect was noted As will be shown, when this between-subject-effect is taken into account a more reliable estimation of the month-effect is obtained

MATERIALS AND METHODS

Immunological assays

Over a period of 12 months (February 1986–January 1987) five serial blood samples were collected from 47 PhD students, 41 males and six females, with an average age of 35 years (s d 6 3, range 26 58) Since there are only two observations in January 1987 this month was excluded from further analysis (see Table 1) All samples were collected during the same 2-h period in the morning in order to limit diurnal variations

In total, 235 (47 × 5) observations per immunological assay were planned Missing observations due to technical errors or missing blood samples were always less than 25 As an exception, for the cell-mediated lympholysis (CML) the results of only 21 subjects were available for statistical analysis (Table 1)

Blood samples

At each sampling point, 40 ml of heparinized blood (for the cellular assays), 4 ml of blood anti-coagulated with ethylenediaminetetraacetic acid (for whole blood cell counts), and 10 ml blood without addition of anti-coagulant (for serum sampling) were collected

From the heparinized blood samples, peripheral blood lymphocytes (PBL) were isolated by Ficoll–Isopaque density gradient centrifugation, washed and resuspended in RPMI 1640 (Dutch modification), supplemented with 3 mmol/*l* L-glutamine, 50 μg/ml gentamycin and 15% heat-inactivated pooled human serum with dimethylsulphoxide (final concentration 10%) for cryopreservation in liquid nitrogen In order to limit the laboratory test variation, all blood samples of a given subject were thawed and tested on the same day in the same experiment The same laboratory supplies were used throughout the whole study

Human leucocyte antigen typing

All subjects, as well as the stimulator cells used in the mixed lymphocyte culture (MLC) and CML assays, were typed for HLA-A, -B and -C antigens with the standard lymphocytotoxicity technique (van Rood, 1974), typing for HLA-DR antigens was performed with the two-colour fluorescence test (van Rood, van Zeeuwen & Ploem, 1976)

Whole blood cell count

Whole blood cell counts and differential white blood cell counts were analysed by a Coulter counter Information was obtained on the concentration of haemoglobin (Hb mmol/*l*), number of erythrocytes (10^{12}/*l*) and the haematocrit volume The mean corpuscular volume (MCV), mean Hb concentration (MCV), and mean corpuscular Hb concentration (MCHC) were computed from the above information Within the population of leukocytes (10^9/*l*) the percentage of eosinophils, basophils, polymorphonuclear cells, neutrophils, lymphocytes and monocytes was assessed Screening was also performed for presence of (pro)myelocytes, meta and blast cells

Phenotype analysis

The mononuclear cells were stained by a standard indirect immunofluorescence technique using the following monoclonal antibodies anti-CD3 (OKT3), anti-CD4 (OKT4), anti-CD8 (OKT8), anti-CD16 (Leu11b and Leu19), anti-TAC (anti-IL-2 receptor) and anti-OKMI (anti-monocytes and -granulocytes) in the first step and goat anti-mouse (GAM) in the second step, and assayed on a fluorescence activated cell sorter (FACS analyser, Becton Dickinson, Mountain View, CA)

Serum levels of immunoglobulins IgG and IgM

Separate serial blood samples were taken to measure IgG and IgM serum concentrations Serum was assayed for albumin, IgG and IgM using a Beckmann array protein system All tests were performed according to the manufacturer's conditions (Sternberg, 1977, Salden *et al*, 1988)

Lymphocyte transformation test (LTT)

Peripheral blood lymphocytes were incubated in tissue culture medium (RPMI 1640 supplemented with 3 mmol/*l* L-glutamine, 50 μg/ml gentamycin and 15% heat-inactivated pooled human serum) for 2 days in the presence of mitogen phytohaemagglutinin (PHA), pokeweed (PWM) or concanavalin A (Con A) To 100 μl cell suspension 100 μl of the following mitogens were added PHA (HA-16, Wellcome Diagnostics, Dartford, UK, 2 mg/5 ml) from a 4 μg/ml dilution, PWM (Gibco, Paisley, Scotland, cat no 061-05360) from a 1/100 dilution, or Con A (Calbiochem Behring Corp, La Jolla, CA, 234567, 2 5 mg/ml) from 10 μg/ml and 40 μg/ml dilutions The mitogen-induced proliferative activity was measured at day 3 after overnight

Table 2. Subject effect and month-effect for all immunological variables

Variable	Subject				Month	
	Between-subject variance	Residual variance	% variance†	Reduction factor‡	*F*	*P*
Haemoglobin	0 3657	0 1094	77	2 1	1 07	0 389
HT	6 53 ^{04}	3 23 ^{04}	67	1 7	1 71	0 082
Erythrocytes	0 1257	0 0615	67	1 7	1 58	0 117
MCV	12 847	5 5041	70	1 8	1 82	0 061
MCH	0 0068	0 0017	80	2 2	2 96	0 002*
MCHC	0 1506	0 2867	35	1 2	2 10	0 027*
Leukocytes	1 0097	0 8265	55	1 5	0 078	0 645
Eosinophils	2 37^{-04}	2 4^{-04}	50	1 4	1 21	0 292
Basophils	0 07 ^{04}	0 28 ^{04}	20	1 1	1 13	0 342
Neutrophils	0 004	0 0044	45	1 3	1 70	0 087
Lymphocytes	0 003	0 0043	41	1 3	1 20	0 299
Monocytes	1 70 ^{04}	5 38^{-04}	24	1 2	1 44	0 170
CD3	63 095	29 755	68	1 8	1 78	0 069
CD4	58 759	34 75	63	1 6	2 77	0 004*
CD8	46 519	11 838	80	2 2	2 82	0 003*
CD4/CD8	0 4303	0 1181	79	2 2	1 70	0 086
α-Tac	19 525	2 9229	87	2 8	0 71	0 716
CD16	51 487	22 192	70	1 8	1 60	0 112
α-monocytes	87 548	35 471	71	1 9	1 57	0 120
IgG	2 9378	0 9904	75	2 0	1 42	0 178
IgM	0 1777	0 0443	80	2 2	1 65	0 099
PHA	0 0145	0 0095	60	1 6	2 27	0 017*
PWM	0 0198	0 0177	53	1 5	2 36	0 013*
Con A 10	0 0296	0 0281	51	1 4	3 10	0 001*
Con A 40	0 0158	0 0172	48	1 4	3 91	0 000*
MLC-1	0 0722	0 0152	83	2 4	3 74	0 000*
MLC-2	0 0537	0 0107	83	2 5	1 60	0 113
MLC-3	0 0905	0 0163	85	2 6	1 28	0 245
CML 1	0 0134	0 0061	69	1 9	0 66	0 760
CML 2	0 0166	0 0067	71	1 9	0 98	0 466
CML-3	0 0176	0 0045	80	2 2	1 37	0 209

* Significant

† Relative between subject-effect

‡ $\sqrt{(\sigma^2_{subj}+\sigma^2_{resid})/\sigma^2_{subj}}$

HT, Haematocrit volume, MCV, mean corpuscular volume, MCH, mean corpuscular haemoglobin, MCHC, mean corpuscular haemoglobin concentration, PHA, phytohaemagglutinin, PWM, pokeweed mitogen, Con A, concanavalin A, MLC, mixed lymphocyte culture, CML, cell-mediated lympholysis

labelling of the cultures with ^{3}H-thymidine The $\log_{10}$ of the counts was taken in order to reduce the effect of extremely high counts

Cell-mediated lympholysis

Allogeneic cytotoxic effector cells were generated in small culture flasks through coculture of 8×10^6 PBL as responder cells with 8×10^6 30 Gy-irradiated stimulator cells in 16 ml culture medium The stimulator cells were derived from three different pools of lymphocytes each consisting of mononuclear cell preparations from three healthy individuals carrying different HLA antigens The responder/stimulator cell combinations were cultured for 6 days and thereafter tested against the three stimulator cell pools as target cells Cytotoxicity was measured using the standard CML assay (Goulmy, 1982) Briefly, 5×10^3 ^{51}Cr-labelled T lymphoblasts, generated by treatment of PBL with 1% PHA mitogen (DIFCO Laboratories, Detroit, MI) for 3 days and expanded in IL-2, were incubated at 37 C for 4 h together with 2×10^5, 1×10^5 and $0\,5\times10^5$ effector cells (E T=40 1, 20 1 and 10 1) The supernatants were then harvested for counting in a gamma counter (Packard Instruments, Downers Grove, USA) The percentage of specific ^{51}Cr-release was calculated according to the following formula

$$\frac{\text{experimental release}-\text{spontaneous release}}{\text{maximal release}-\text{spontaneous release}}\times 100\%$$

Mixed lymphocyte culture

Primary cultures were established in round-bottomed microtitre plates Responder cells (10^5) were incubated for 5 days with 10^5 irradiated (30 Gy) stimulator cells derived from three different pools of lymphocytes, each consisting of mononuclear cell preparations from three healthy individuals carrying different HLA antigens Sixteen hours before harvesting, the cultures

were labelled with 1 μCi ^{3}H-thymidine Isotope incorporation was measured in a liquid scintillation counter Experimental values are computed as the $\log_{10}$ of the median of the results of the triplicate cultures expressed in counts per min (ct/min)

Statistical analysis

In order to correct for the imbalance due to unequal distribution of the data over the months and to take into account the between-subject variation, the immunological data were analysed using (SPSS-X) MANOVA, multivariate analysis of variance, with a two-way factorial, subject × month, design with 47 subject and 11 month categories

Our data can be described as $x_j = \mu + \alpha + \beta_j + e_{ij}$, in which x is the raw score for subject i at time j, μ is the group mean, α is the between-subject-effect, β is the month-specific-effect and e the residual (error) The between-subject variance (σ^2_{subj}) is the variance of the α_i's and the residual variance (σ^2_{resid}) is the variance of the e_{ij}'s Analysis of variance is used for two purposes, firstly to test for a month-effect and secondly to estimate the two variance components between-subject and residual variance, respectively (To be complete we also performed a formal significance test for the presence of between-subject variance) These variance components can be estimated by classical methods as described by Fleiss (1986) Having computed the two variance components, two related quantities are of interest Firstly, the percentage of the total variance that is due to the between-subject variation ($100 \times (\sigma^2_{subj}/(\sigma^2_{subj} + \sigma^2_{resid})$) and secondly the reduction factor by which the standard error of the estimated month-effect is reduced when the subject effect is corrected for This reduction factor applies when comparing the effects for different months Moreover, to check that the estimate of the month effect was not affected by the intervention, two tests on the intervention-effects were carried out by the MANOVA with sequential subject intervention and month-effect, where the intervention was assumed to have effect either 2 weeks before or 2 weeks after (test 1) or 1 month before or 1 month after (test 2) the defence of the PhD thesis Furthermore, an example is given of the misleading effect of deleting the subject-effect in the analysis and the merits of our alternative approach in which the between-subject effect is taken into account

RESULTS

Reduction of the standard error of month-effects by taking into account the between-subject variability

The variance components and related quantities are given in Table 2 All between subject variances were highly significant ($P < 0.001$ for all variables considered) From Table 2 it is apparent that the relative subject variance is > 70% for 16 of the variables studied This is equivalent to a reduction factor > 1 8 for the standard error of the estimated month effect To illustrate the effect we have presented some detailed results for CD8 In Fig 1a the mean percentage of CD8 cells per month and the standard errors are given and in Fig 1b the estimated month-effect after correction for the subject-effect and the corresponding standard errors are given The standard errors in Fig 1b are about twice as small as in Fig 1a which is in accordance with the reduction factor given in Table 2

All results presented below are corrected for the between subject effect as described above

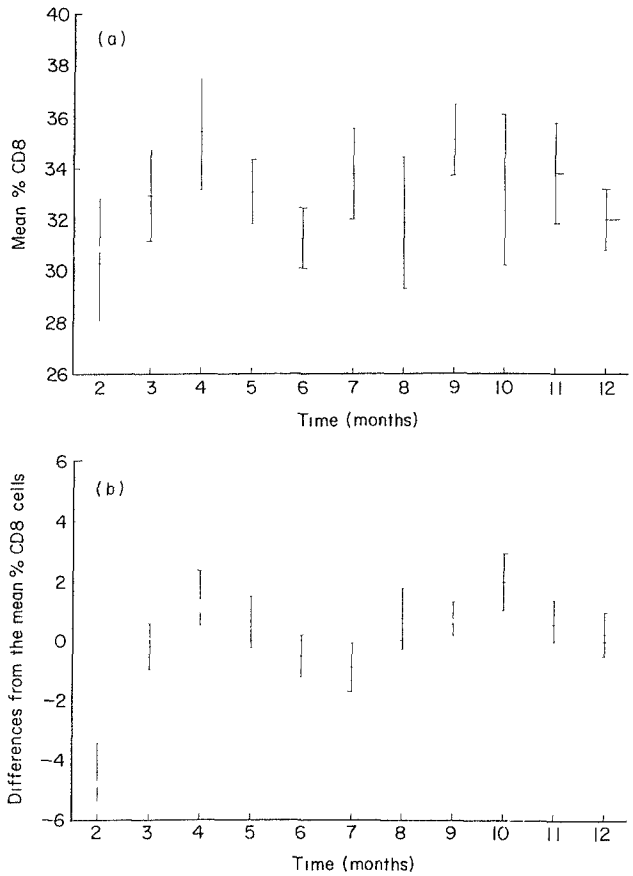

Fig 1 (a) The mean percentage of CD8 cells per month (±s e) The standard errors of the monthly averages are uncorrected for the between subject variance (b) Percentage of CD8 cells per month (±s e) The results are expressed as the differences per month from the overall mean percentage of CD8 cells The corresponding standard errors of the estimated month effect are corrected for the between subject variance

Intervention effect

Since the intervention (i e the stress of the defence of a PhD thesis) could influence the results on the month-effects, its effect was established with MANOVA No intervention effect was observed for intervention tests 1 and 2 (see Materials and Methods) (data not shown) The observed month-effects could not therefore be the result of the intervention

Month-effects on cellular and serological immunological measurements

Multivariate analyses of variance of the red and white blood cell counts revealed significant month-of-data-sampling-effects for the MCH ($F = 2.96$, $P = 0.002$, df = 10,119) and the MCHC ($F = 2.10$, $P = 0.027$, df = 10,119) (Table 2) The month-effect for MCH was found in February, March and October and for MCHC in February and October (Table 3)

Multivariate analyses of variance of the results of phenotype analyses revealed a significant month-effect for the percentage of CD4 ($F = 2.77$, $P = 0.004$, df = 10,145) and CD8 cells ($F = 2.82$, $P = 0.003$, df = 10,145), but not for the CD4/CD8 ratio, the percentage of CD3 Leu11 and Leu19 (CD16) cells and for the percentage of cells staining with the anti-TAC monoclonal antibody (Table 2) The month-effect was found for CD4

Table 3. Mean, standard error and differences from the mean per month for the immunological variables for which a significant month-effect was observed

	MCH	MCHC	CD4	CD8	PHA	PWM	Con A 10	Con A 40
				Mean				
Month	1 842	20 520	55 108	33 275	5 100	4 394	4 232	4 632
2	0 023*	0 286*	−2 548	−4 400*	−0 007	−0 054	−0 017	−0 015
3	−0 028*	−0 038	−1 853	−0 213	0 023	0 017	0 032	0 008
4	−0 010	−0 221	−1 344	1 424	−0 080*	−0 003	−0 059	−0 080*
5	−0 002	−0 202	−3 316*	0 616	−0 003	−0 032	−0 029	−0 027
6	−0 002	−0 137	−0 097	−0 534	0 010	−0 009	0 006	0 008
7	−0 009	−0 035	−1 490	−0 912	0 004	−0 056	−0 024	−0 042
8	−0 015	0 270	1 132	0 730	−0 017	−0 045	−0 141*	−0 091*
9	−0 008	−0 209	0 633	0 530	−0 031	−0 025	−0 048	−0 032
10	0 031*	0 330*	3 090*	1 966*	0 015	0 081*	0 046	0 061
11	0 017	0 098	5 400*	0 549	0 078*	0 112*	0 165*	0 143*
12	0 003	−0 142	0 396	0 243	0 007	0 012	0 070*	0 067*
s e	0 008 0 012	0 110–0 160	1 21 1 76	0 740 1 03	0 020 0 029	0 027 0 040	0 035 0 048	0 027 0 041

* Significant

Values are differences from the overall mean

s e, Range of standard error, MCH mean corpuscular haemoglobin, MCHC mean corpuscular haemoglobin concentration, PHA phytohaemagglutinin, PWM pokeweed mitogen, Con A, concanavalin A

in May, October and November and for CD8 in February and October (Table 3)

For the serum concentrations of immunoglobulins IgG and IgM no significant month-effect was observed (Table 2)

Month-effects on functional immunological in vitro studies

All mitogenic responses tested in our study had a significant month effect PHA ($F=2\ 27$, $P=0\ 017$, df = 10,145), PWM ($F=2\ 36$, $P=0\ 013$, df = 10,145), Con A 10 μg/l ($F=3\ 10$, $P=0\ 003$, df = 10,145) and Con A 40 μg/l ($F=3\ 91$ $P=0\ 000$, df = 10,145) (Table 2) The month-effect was found for PHA in April and November, for PWM in October and November for Con A 10 in August, November and December and for Con A 40 in April, August, November and December (Table 3)

The results for the primary MLC were inconsistent Of the three pools tested, only MLC-1, but not MLC 2 and MLC-3 was observed to have a significant month-effect ($F=3\ 74$, $P=0\ 000$, df = 10,155) (Table 2) Comparison of HLA frequencies in the three pools did not reveal any differences which could explain this inconsistency However the standard deviation of the triplicates was quite often above 25% (in 40% of the observations) This was twice as often the case for pool 1 as for pool 2 and 3 A possible explanation for this latter finding could be that the routine plating of this pool, i e the first row of a microtitre test plate, has led to a systematic error

No significant month-effect was observed on cytotoxic T cell alloreactivity measured against any of the three stimulator pools used (Table 2)

DISCUSSION

Two sources of variance are described which if they are not accounted for, can seriously interfere with a reliable interpretation of the results of immunological follow up studies The between-subject variability accounts in our study for about 70% of the total variance Since the between-subject-effect is so dominant, it implies that very large sample sizes are needed to find significant differences between groups *if only a single* observation per individual is available However if the same group of subjects is repeatedly, and preferably more than twice measured for the same immunological parameters then the between-subject variability is easily taken into account and other sources of variation including that which is related to month of-data-sampling, can be estimated with greater accuracy In our study the standard error of the month-specific values was in this way decreased by a factor of about 2

The variability in immunological *in vitro* data which is related to the month of-data-sampling is less easily established in an intervention study It is impossible to estimate the month-effect independent of the intervention-effect if the intervention or treatment is implemented on a specific date or in a specific month However, if a control group is tested in parallel to the experimental group then the results of the control group can be used to estimate the month-related variability In an ongoing longitudinal study like ours in which the date of intervention varies the month-effect can also be established An equal distribution of date of intervention over the months of the year would be the most complete and graceful design, resulting in the most accurate estimation of the month-effect and therefore also in the most accurate estimation of the intervention-effect The possibility of faulty conclusions if the month-related variability is not taken into account can best be demonstrated with an example If, in our study, the percentage of CD4 cells was assessed pre-treatment in May and the post-treatment data were collected in October and November then a significant effect would have been observed (Table 3) This significant effect would, in the absence of a control group be attributed to the treatment When the month-related variability is taken into account it becomes evident that the observed significant effect can be as well or better accounted for by the month related

variability Special attention is therefore needed when, in follow-up studies, immunological assays are used for which significant month-effects have been observed

We observed month-effects for MCH and MCHC, the percentage of CD4 and CD8 cells and for the proliferative response to PHA, PWM and Con A These results confirm and extend previous results by others who observed a month-effect for MCH and MCHC (Rocker *et al*, 1980), for the number of leukocytes, T cells and B cells (Bratescu & Teodorescu, 1981, MacMurray *et al*, 1983), for the proliferative response to mitogens (Boctor *et al*, 1989) and for serum concentrations of IgG (Lyngbye & Kroll, 1971, MacMurray *et al*, 1983)

These results cannot be used to correct results of the same immunological assays obtained in another year or climatological area However, the statistical method presented can be of use for others since it allows the estimation of the month-effect in a reliable way, i e after taking the between-subject-effect into account Inference about the statistical significance of the effect of the intervention can be derived from the comparison of the *I*-values of month-effect and intervention-effect An important advantage of the presented method is that it can easily be applied in different kinds of immunological follow-up studies

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