British Journal of Nutrition (2003), **89**, 695–703
DOI: 10.1079/BJN2003826

Zinc supplementation has no effect on circulating levels of peripheral blood leucocytes and lymphocyte subsets in healthy adult men

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(Received 9 April 2002 – Revised 11 November 2002 – Accepted 16 December 2002)

As a result of evidence documenting harmful effects of Zn supplementation on immune function and Cu status, thirty-eight men were recruited onto a Zn supplementation trial. The aim was to examine the effects of chronic Zn supplementation on circulating levels of peripheral blood leucocytes and lymphocyte subsets. Subjects (*n* 19) took 30 mg Zn/d for 14 weeks followed by 3 mg Cu/d for 8 weeks to counteract adverse effects, if any, of Zn supplementation on immune status resulting from lowered Cu status. A control group (*n* 19) took placebo supplements for the duration of the trial. Dietary intakes of Zn approximated 10 mg/d. Blood samples, taken throughout the trial, were assessed for full blood profiles and flow cytometric analyses of lymphocyte subsets. Putative indices of Cu status were also examined. Results indicate that there was no effect of Zn supplementation on circulating levels of peripheral blood leucocytes or on lymphocyte subsets. Cu status was also unaltered. Independent of supplement, there appeared to be seasonal variations in selected lymphocyte subsets in both placebo and supplemented groups. Alterations in circulating levels of B cells (cluster of differentiation (CD) 19), memory T cells (CD45RO) and expression of the intracellular adhesion molecule-1 (CD54) on T cells were observed. Findings indicated no adverse effects of Zn supplementation on immune status or Cu status and support the US upper level of Zn tolerance of 40 mg/d. The seasonal variations observed in lymphocyte subsets in the group as a whole could have implications for seasonal variability in the incidence of infectious diseases.

Zinc: Leucocytes: Lymphocyte subsets: Immune status

The essentiality of Zn for man was first documented by Prasad and colleagues in the 1960s (Prasad *et al.* 1963). Zn is a cofactor of more than 300 enzymes (Rink & Kirchner, 2000) and is needed for growth, normal development, DNA synthesis, immunity, neurosensory function and other important cellular processes (Wood, 2000). Zn is so ubiquitous in cellular metabolism that even minor impairment of an adequate supply is likely to have multiple biological and clinical effects (Hambidge, 2000). Deficiency of Zn affects the epidermal, gastrointestinal, central nervous, immune, skeletal and reproductive systems (Hambidge & Walravens, 1982). Some studies suggest that sub-optimal Zn might be prevalent in human populations (Walravens *et al.* 1989; Sandstead, 1995; Brown *et al.* 1998).

These observations and others have led to the advocacy of Zn supplements for the prevention and management of a variety of disorders. Zn administration is the standard therapy for acrodermatitis enteropathica (a genetic defect in the ability to assimilate Zn), and is given therapeutically to compete with Cu for absorption in Wilson's disease. Additionally, Zn supplementation has been used successfully to restore immune function in human subjects, particularly in elderly individuals (Duchateau *et al.* 1981; Boukaïba *et al.* 1993; Prasad *et al.* 1993).

Excessive Zn intake, however, has the potential to cause adverse effects (Chandra, 1984; Fosmire, 1990). Acute toxicity of Zn causes nausea and vomiting, epigastric pain, abdominal cramps and diarrhoea (Brown *et al.* 1964). Of more relevant concern, is evidence that high doses of Zn can adversely affect immune functioning and Cu status. Schlesinger *et al.* (1993) demonstrated that supplementing marasmic infants during nutritional rehabilitation with Zn

Abbreviations: CD, cluster of differentiation; FITC, fluorescein; HLA-DR, human leucocyte antigen, DR locus; ICAM, intracellular adhesion molecule; NOAEL, no-observed-adverse-effect level; PE, phycoerythrin.

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(1·9 mg/kg per d) for 105 d exerted an inhibitory effect on phagocytic and fungicidal capacity of monocytes as well as promoting a higher incidence and duration of impetigo episodes in these children. Zn supplementation of 300 mg/d for 6 weeks in healthy adult men impaired chemotaxis and phagocytosis of bacteria by polymorphonuclear leucocytes (Chandra, 1984). Some of the immunological effects of Zn supplementation might be through interference with Cu status. For example, erythrocyte superoxide dismutase activity was significantly reduced in females who took 50 mg Zn/d for 10 weeks (Yadrick *et al.* 1989). Decreases in serum Cu concentrations have been observed in institutionalised elderly subjects supplemented with 20 mg Zn/d for 8 weeks (Boukaïba *et al.* 1993). Additionally, two studies have found that Zn supplementation at doses of 75 mg/d for 12 weeks (Black *et al.* 1988) and 160 mg/d (Hooper *et al.* 1980) for 6 weeks, decreased concentrations of HDL-cholesterol in male subjects. Increases in serum cholesterol are a consequence of Cu deficiency in growing animals (Underwood, 1977; Allen & Klevay, 1994) and changes in cholesterol concentration have also been observed in human subjects during studies of short-term mild Cu deprivation (Klevay *et al.* 1984; Nielsen *et al.* 1990). The reported effects of Zn supplementation on Cu status, therefore, may have wide-reaching consequences.

The tolerable upper level for Zn intake in the USA is set at 40 mg/d (Institute of Medicine, Food and Nutrition Board, 2001), based on experiments that examined the adverse effects of excess Zn on Cu metabolism (Fischer *et al.* 1984; Samman & Roberts, 1988; Yadrick *et al.* 1989). Work examining the effect of the no-observed-adverse-effect level (NOAEL) for Zn on indices of immune status has not been reported. The present study investigated the acute (for 2 weeks) and chronic (for 12 weeks) effects of Zn supplementation (30 mg/d) in healthy males on circulating levels of blood leucocytes and phenotypic expression of lymphocyte subsets. In particular, T lymphocyte subsets involved with T cell activation were examined. As there are concerns regarding the possible effect of Zn supplementation on Cu status, subjects were supplemented with Cu (3 mg/d for 8 weeks) after Zn supplementation.

Materials and methods

Subjects

Male subjects (mean (SD) age 35·6 (9·6) years) were recruited to participate in the study. All subjects were initially screened via a blood sample, which was analysed for full blood picture, liver enzymes and lipid profile. Subjects with values for blood, lipid or liver enzyme profiles outside established ranges were excluded. Additionally, subjects were screened by a lifestyle questionnaire and were eligible for inclusion if they were non-smokers, had no acute or chronic illness, were taking no medication or vitamin supplements and had a BMI less than 30 kg/m^2. A total of forty subjects initially started the trial and thirty-eight completed the trial. The study protocol was reviewed and approved by the Research Ethical Committee of the University of Ulster.

Experimental design

The study design was a double-blinded intervention trial with two randomly selected groups, each of twenty subjects. The test group was supplemented with 30 mg Zn/d as Zinc Chelazome® (zinc glycine chelate) for 14 weeks followed by 3 mg Cu/d as Copper Chelazome® (copper glycine chelate) for 8 weeks to counteract adverse effects, if any, of Zn supplementation. The other group took placebo for the full duration of the trial. Supplements were supplied by Thomson and Joseph Ltd (Norwich, UK). The study design necessitated the staggering of blood sampling. Sampling commenced in October 1999 and was completed in May 2000. Sampling time points were weeks 0, 2, 14, 16, 18 and 22.

Habitual dietary assessment

Habitual dietary information was collected from each subject by means of a 4 d dietary record (two week days and both weekend days) at the beginning and on completion of the trial. Dietary records were analysed using standard food portion sizes (Crawley, 1992) and nutrient intake calculated using the nutrient database package WISP (Tinuviel Software, Warrington, UK). A self-administered lifestyle questionnaire was used to obtain information on alcohol consumption, occupation, exercise and medical history as well as average intake of Cu-rich foods.

Collection and preparation of samples

Blood samples (45 ml) were collected after an overnight fast by antecubital venepuncture into either evacuated heparinised, EDTA or serum tubes (vacuette, greival bio-one, Austria) at baseline and at the end of weeks 2, 14 (end of Zn supplementation period), 16, 18 and 22 (end of Cu supplementation period). An EDTA blood sample was sent to Causeway Health and Social Services Trust Laboratories (Coleraine, UK) at each time point for analysis of full blood profiles. A sample of whole blood (heparinised tubes) was stored at −80°C for whole blood superoxide dismutase activity. The remainder of the heparinised samples were centrifuged (717 ***g*** for 15 min at 4°C) and 200 μl plasma samples were stored at −80°C for caeruloplasmin oxidase activity. Serum blood samples were left to clot for 1 h and then centrifuged at 1614 ***g*** for 10 min. Samples of serum were stored at −80°C for analysis of caeruloplasmin protein and assessment of serum Cu and Zn concentrations. Whole blood from EDTA tubes was processed for flow cytometric analysis on the day of blood taking.

Total leucocyte and differential leucocyte counts

Total leucocyte and differential leucocyte counts were performed on a CELL-DYN 3500 (Abbott Laboratories, Abbott Park, IL) within 4 h of venepuncture. Low, normal and high quality-control samples were run each morning at a laboratory participating in the UK National External Quality Assessment Scheme.

Analysis of cell surface markers by flow cytometry

Antibodies and all other reagents were supplied by Becton Dickinson (Immunocytometry Systems, Oxford, UK). Antibodies were either labelled with fluorescein (FITC) or phycoerythrin (PE). The following combinations of dual-labelled monoclonal antibodies or combinations of single antibodies labelled with FITC or PE were used: immunoglobulin G1 FITC–immunoglobulin G2a PE isotype control; cluster of differentiation (CD) 3 FITC–CD19 PE; CD3 FITC–CD16+CD56 PE; CD3 FITC–CD4 PE; CD3 FITC–CD8 PE; CD3 FITC–HLA-DR PE; CD3 FITC–CD45RO PE; CD3 FITC–CD25 PE; CD3 PE–CD45RA FITC; CD3 PE–CD54 FITC; CD3 PE–CD11a FITC. All combinations were measured on lymphocytes. Whole blood (100 μl) was incubated with 10 μl (dual-labelled antibodies) or 20 μl (10 μl each of the single-labelled antibodies) for 15 min, in the dark, at room temperature. Cells were then lysed by adding FACS lysing solution (2 ml) and left for 10 min, in the dark, at room temperature. Following lysis, samples were centrifuged at 717 ***g*** for 5 min at room temperature to remove lysed erythroctes and then washed twice with CellWASH. Washed cells were re-suspended in CellFIX (500 μl) and analysed within 4 h. All samples were analysed using a FACScalibur flow cytometer calibrated weekly with CaliBRITE beads and AutoCOMP software (Becton Dickinson). Negative control antibodies were used to separate positive and negative cells on both the FITC and PE channels for every subject at each blood sampling point. Compensation settings on controls were kept constant for all other samples for each participant. Lymphocyte gates were also determined from the negative control and set up to collect 10 000 events per sample. Analysis was performed using Cellquest software (Becton Dickinson). The sum of the three lymphocyte populations expressed as a percentage (CD3+, CD19+ and CD3-(CD16+56) i.e. T + B + natural killer cells) was calculated for each subject at each time point as a further quality-control procedure and should approximate 100 ± 5 %. At baseline total T, B and natural killer cells were 96·67 % and on conclusion of the trial total T, B and natural killer cells were 95·37 % (data not shown).

Caeruloplasmin oxidase activity and caeruloplasmin total protein concentration

Plasma caeruloplasmin oxidase activity was determined by a modification of the method of Henry *et al.* (1960), using *p*-phenylenediamine dihydrochloride (Sigma Aldrich Co Ltd, Poole, Dorset, UK) as substrate and measuring the rate of oxidation of *p*-phenylenediamine dihydrochloride at 37°C. Analysis was performed, in duplicate, on the Cobas Fara automatic analyser (Roche, Basel, Switzerland). Serum caeruloplasmin protein concentrations were measured turbidimetrically using a modification of the method of Calvin & Price (1986) on the Hitatchi 912 (Roche). All serum and standard dilutions were carried out by the Hitatchi 912. Samples were mixed with excess rabbit anti-human caeruloplasmin (Dako, Glostrup, Denmark), the absorbance at 340 nm was measured and concentrations (U/l) determined from a standard curve calculated using a human serum protein calibrator (Dako).

Whole blood superoxide dismutase activity

The activity of whole blood superoxide dismutase was determined in duplicate on the Hitachi 912 (Roche Diagnostics) by a modification of the method of Jones & Suttle (1981), using a commercial kit, RANSOD (Randox Laboratories, Co. Antrim, UK). Absorbance at 505 nm was measured (reference wavelength 700 nm) and the superoxide dismutase activity of samples were determined from a standard curve and expressed as U/ml whole blood.

Serum copper and zinc

Serum Zn and Cu were measured by flame atomic absorption spectrophotometry (spectrophotometer model AA6701 Shimadzu autosampler; Mason Technology, Dublin, Republic of Ireland). Cu and Zn standard solutions (Sigma Aldrich, Poole, Dorset) and low, normal and elevated multi-sera (Randox Laboratories, Co. Antrim, UK) were run alongside samples. Briefly, Cu and Zn samples were acid-digested with 0·5 ml HCl (3 M; Merck Eurolab Ltd, Poole, Dorset, UK) for 10 min, followed by 0·5 ml TCA (40 %, w/v) (Merck Eurolab Ltd) for a further 10 min. Deionised water (3 ml) was then added to the samples which were allowed to sit for a further 10 min. Samples were then centrifuged for 10 min at 3623 ***g*** and the supernatant fractions analysed (method according to manufacturers manual) at absorbances 324·8 and 213·9 nm for Cu and Zn respectively.

Statistics

All data were analysed using the SPSS 9·0 for Windows statistical package (SPSS Inc., Chicago, IL). Data for all variables were tested for normality and adjusted where necessary. Differences between baseline and week 14 were compared across groups using the independent *t* test to determine effects of Zn supplementation. This was repeated between weeks 14 and 22 to examine for any effect of Cu supplementation. Effect of time and supplement were analysed by repeated measures ANOVA (and any interaction between time and supplement noted).

For a number of variables there was a temporal alteration occurring in both groups that was independent of supplement (no time–supplement interaction observed). To examine this observation, data from placebo and supplement groups were pooled and paired *t* tests using Bonferroni's correction for multiple comparisons were used to identify at what time point/s the temporal effect was present. A significance level of $P<0{\cdot}05$ was used.

Results

A total of thirty-eight subjects completed the trial. Subject characteristics and dietary data at the start of the trial are illustrated in Table 1. There was no significant difference between groups at baseline in any of the variables shown in Table 1.

Table 1. Subject characteristics and dietary profiles at baseline (Mean values with their standard errors)

	Placebo group (*n* 19)		Supplement group (*n* 19)	
	Mean	SE	Mean	SE
Age (years)	35·26	2·20	35·84	2·36
BMI (kg/m^2)	25·32	0·70	25·96	0·65
Energy (kJ/d)	9669	368	10652	531
Dietary Cu (mg/d)	1·10	0·09	1·28	0·06
Dietary Zn (mg/d)	9·56	0·78	9·29	0·51

There was no effect of Zn or Cu supplementation on serum Zn and serum Cu concentrations, caeruloplasmin oxidase activity, caeruloplasmin protein concentrations or whole blood superoxide dismutase concentrations (Table 2). Independent *t* tests at baseline on both differential blood counts (data not shown) and flow cytometric data (Table 3) indicated no difference between groups at the beginning of the trial in any of the parameters studied, except for CD45RA. Lower levels of circulating T cells (CD3) expressing CD45RA ($P=0·042$) were seen in the placebo group compared with the supplemented group at baseline. This difference is possibly reflected by a lower, but not significant, difference in CD3 (particularly the CD8 subset) numbers at baseline in the placebo group compared with the control group. HLA-DR expression appeared to be lowered after 14 weeks of Zn supplementation compared with control, but results did not attain statistical significance.

No effect of Zn supplementation (nor Cu supplementation) was seen on any of the indices examined (Table 3). However, a number of corresponding changes in lymphocyte subsets (absolute numbers) were observed simultaneously in both placebo and supplement groups. This effect was not an effect of supplement, as it was similarly observed in both groups. To examine this observation further, placebo and supplement data were pooled and re-analysed by repeated measures ANOVA and Bonferroni's correction for multiple comparisons (Table 4).

Flow cytometric analysis indicated significant alterations over the period of the trial in three parameters. This observation was independent of supplement (see *P* value in Table 3) and this effect of time was evident when data were expressed as absolute numbers (Table 4) and also when the cell subsets were analysed as a proportion (%) of cells within the lymphocyte lineage (data not shown). A significant decrease in CD19 (B cells) was observed between week 0 and 14 ($P=0·00$) and week 2 and 14 ($P=0·000$) and significant increases were observed between weeks 14 and 16 ($P=0·030$) and week 14 and 18 ($P=0·045$). Expression of CD3+/CD45RO+ (T memory cells) decreased throughout the trial with decreases between week 0 and 14 ($P=0·000$), week 0 and 18 ($P=0·000$), week 2 and 14 ($P=0·015$) and week 2 and 18 ($P=0·030$). Lastly, expression of CD3+/CD54+ (intracellular adhesion molecule (ICAM)-1) decreased significantly between week 0 and 14 ($P=0·000$) and increased significantly between week 14 and 22 ($P=0·000$).

Discussion

The present study was undertaken to evaluate the effects of Zn supplementation on selected immunological measures in healthy adult male subjects. Total Zn intake (including supplements of 30 mg Zn/d) was approximately 40 mg/d for the subjects on Zn supplements. When designing the trial, it was thought probable that some interference of Cu absorption at this level of Zn supplementation might occur without an appreciable concomitant depletion of other nutrients, including Fe, and without alterations in traditional markers of Cu status, such as caeruloplasmin. Previous studies have shown that slightly higher doses (50 mg Zn/d) altered indices of Cu status (Fischer *et al.* 1984; Yadrick *et al.* 1989) over a shorter period of time. Currently, the lowest observed adverse effect level in the USA for dietary Zn intake is 60 mg/d, with the adverse effect attributed to interference with Cu metabolism (Institute of Medicine, Food and Nutrition Board, 2001). A tolerable upper level for Zn was established at 40 mg/d (also the value chosen for the NOAEL) but it has been argued that the maximal safe dose of supplemental Zn is unknown (Sandstead & Smith, 1996).

Cu intakes in placebo and supplemented groups (1·10 and 1·28 mg/d respectively) appeared to be adequate based on the reference nutrient intake value of 1·2 mg/d (Department of Health, 1991). Mean estimated dietary intakes of Zn in the placebo group (9·29 mg/d) were just below the reference nutrient intake of 9·5 mg/d and Zn intakes in the supplement group were adequate (9·56 mg/d). Subject compliance was optimised by the use of 14 d supplement packs which could be monitored for usage and were replaced fortnightly. Measurement of Zn status was not considered as a method of monitoring compliance because clinically available, highly specific biochemical markers of Zn status do not exist (Sandstead & Smith, 1996).

Results indicate that the traditional markers of Cu status, caeruloplasmin oxidase activity, serum caeruloplasmin protein concentrations and whole blood superoxide dismutase concentrations, were unaltered by Zn supplementation followed by Cu supplementation (data not shown). Levels of circulating leucocytes (full blood profile) were also unaffected by Zn supplementation. Phenotypic expression of lymphocytes (T cells, B cells and natural killer cells) and T cell lymphocyte subsets (CD4 and CD8) fell within published reference ranges (Reichert *et al.* 1991) and were unaltered by Zn supplementation. Zn supplementation also had no effect on the following T lymphocyte subsets: two markers of T-cell activation CD25 and HLA-DR; the adhesion molecules CD11a and CD54; naive and memory T cells (CD45RA and CD45RO respectively). Measurement of these markers on T lymphocytes gives an indication of T cell function, which has been observed to be impaired as a result of Cu deficiency in man (Kelley *et al.* 1995) and laboratory animals (Bala *et al.* 1991).

A reduction in the number of circulating leucocytes in the peripheral blood has been noted in elderly patients with marginal Zn status (Kaplan *et al.* 1988) and Zn supplementation (440 mg zinc sulfate/d for 1 month) was observed to increase circulating numbers of T-lymphocytes in fifteen elderly subjects (Duchateau *et al.* 1981). These data contrast

Table 2. Effect of zinc supplementation (followed by copper repletion) on putative indices of copper and zinc status in healthy men*
(Mean values with their standard errors)

	Baseline		Week 2		Week 14		Week 16		Week 18		Week 22		
	Placebo	Active	Placebo	30 mg Zn	Placebo	30 mg Zn	Placebo	30 mg Cu	Placebo	30 mg Cu	Placebo	30 mg Cu	
Putative indices of Cu and Zn status	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	*P* value†
Ceruloplasmin oxidase activity (U/l)	543·10 17·74	554·59 19·75	583·54 21·50	564·06 21·17	561·18 17·87	554·02 19·99	549·94 21·61	534·52 20·36	576·09 26·90	554·71 21·72	594·36 23·23	563·16 22·47	0·625
n	19	19	19	19	19	19	19	19	19	19	18	19	
Ceruloplasmin protein (g/l)	0·22 0·01	0·23 0·01	0·22 0·01	0·22 0·01	0·21 0·01	0·21 0·01	0·22 0·01	0·22 0·01	0·23 0·01	0·22 0·01	0·20 0·01	0·22 0·01	0·991
n	19	19	19	19	19	19	19	19	19	19	18	19	
Whole blood superoxide dismutase (U/ml)	187·39 11·57	172·37 6·16	140·22 4·50	141·18 4·02	179·83 9·96	189·02 11·16	179·56 12·14	196·94 11·65	144·26 6·12	156·83 5·37	148·26 4·99	150·54 5·94	0·386
n	19	19	19	19	19	19	19	19	19	19	18	19	
Serum Cu (μg/ml)	0·93 0·06	0·86 0·04	0·98 0·06	0·87 0·04	0·93 0·05	0·88 0·03	0·95 0·07	0·82 0·04	0·93 0·05	0·89 0·05	0·93 0·05	0·91 0·05	0·433
n	17	18	18	18	18	16	16	15	18	17	18	18	
Serum Zn (μg/ml)	1·02 0·39	1·00 0·30	0·92 0·35	1·15 0·47	1·06 0·60	1·26 0·48	0·99 0·44	1·06 0·47	1·03 0·54	1·14 0·62	1·08 0·61	1·27 0·80	0·358
n	17	16	18	16	18	17	16	15	18	17	18	17	

* For details of subjects and procedures, see Table 1 and p. 696.
† Analysed by repeated measures ANOVA with supplement as between-subject factor.

Table 3. Effect of zinc supplementation (followed by copper supplementation) on absolute numbers of lymphocyte subsets in healthy men†
(Mean values with their standard errors)

	Baseline		Week 2		Week 14		Week 16		Week 18		Week 22		
	Placebo	Active	Placebo	30 mg Zn	Placebo	30 mg Zn	Placebo	30 mg Cu	Placebo	30 mg Cu	Placebo	30 mg Cu	
Surface marker (10^9 cells/l)	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE	*P*‡
CD3+(T cells)	1·28 0·06	1·43 0·09	1·27 0·06	1·48 0·10	1·28 0·08	1·37 0·10	1·22 0·07	1·38 0·09	1·16 0·07	1·42 0·09	1·22 0·06	1·32 0·08	0·307
n	18	19	19	18	19	19	19	18	19	19	17	18	
CD19+(B cells)	0·21 0·02	0·20 0·01	0·20 0·01	0·21 0·02	0·17 0·01	0·16 0·01	0·19 0·02	0·18 0·02	0·18 0·01	0·19 0·02	0·18 0·01	0·17 0·02	0·594
n	18	19	19	18	19	19	19	19	19	19	18	19	
CD3-CD(16+56)+(natural killer cells)	0·37 0·06	0·39 0·05	0·38 0·04	0·36 0·04	0·41 0·05	0·37 0·05	0·39 0·05	0·38 0·05	0·35 0·03	0·35 0·05	0·36 0·06	0·34 0·04	0·601
n	18	19	19	18	19	19	19	19	17	18	16	17	
CD3+CD4+(helper T cells)	0·78 0·04	0·77 0·04	0·76 0·03	0·82 0·06	0·77 0·04	0·77 0·06	0·74 0·04	0·76 0·04	0·72 0·04	0·80 0·04	0·75 0·03	0·78 0·06	0·709
n	17	19	19	19	19	19	19	19	19	19	19	19	
CD3+CD8+(cytotoxic T cells)	0·49 0·04	0·61 0·09	0·51 0·04	0·59 0·08	0·51 0·05	0·59 0·08	0·49 0·05	0·60 0·07	0·44 0·04	0·60 0·08	0·47 0·04	0·56 0·06	0·270
n	18	18	19	19	19	19	19	18	19	19	19	19	
CD3+CD45RA+(naive T cells)	0·49* 0·04	0·67 0·07	0·54 0·04	0·65 0·07	0·53 0·04	0·61 0·08	0·51 0·04	0·64 0·07	0·47 0·04	0·62 0·06	0·50 0·03	0·60 0·06	0·193
n	19	18	17	17	19	19	19	19	18	19	19	19	
CD3+CD45RO+(memory T cells)	0·82 0·06	0·76 0·05	0·77 0·05	0·81 0·05	0·73 0·06	0·66 0·04	0·75 0·05	0·68 0·05	0·63 0·04	0·71 0·05	0·73 1·95	0·69 0·04	0·669
n	17	18	18	19	19	19	19	18	19	19	19	19	
CD3+CD25+(interleukin-2 receptor)	0·40 0·03	0·35 0·03	0·37 0·02	0·37 0·03	0·32 0·03	0·29 0·02	0·41 0·06	0·28 0·03	0·30 0·03	0·36 0·03	0·37 0·03	0·38 0·03	0·261
n	17	17	18	19	19	19	18	18	19	19	19	19	
CD3+HLA-DR (activation marker)	0·15 0·02	0·17 0·02	0·15 0·02	0·16 0·02	0·14 0·02	0·13 0·02	0·14 0·02	0·14 0·02	0·11 0·01	0·15 0·02	0·14 0·02	0·15 0·02	0·558
n	17	18	19	19	19	19	18	19	18	19	19	19	
CD3+CD54+(ICAM-1)	0·40 0·03	0·52 0·07	0·37 0·02	0·44 0·05	0·32 0·03	0·39 0·06	0·41 0·06	0·38 0·06	0·30 0·03	0·53 0·07	0·37 0·03	0·54 0·07	0·660
n	16	18	17	17	19	17	17	16	19	19	19	19	
CD3+CD11a+(LFA-1)	1·30 0·07	1·43 0·10	1·31 0·07	1·51 0·11	1·31 0·08	1·39 0·10	1·28 0·08	1·39 0·09	1·18 0·07	1·46 0·09	1·26 0·06	1·36 0·08	0·248
n	18	19	17	17	19	19	18	19	19	18	19	19	

CD, cluster of differentiation; HLA-DR, human leucocyte antigen, DR locus; ICAM, intracellular adhesion molecule; LFA, leucocyte function-associated.
*Significant difference at baseline ($P<0·05$).
† For details of subjects and procedures, see Table 1 and p. 696.
‡ Analysed by repeated measures ANOVA with supplement as between subject factor.

Table 4. Effect of season on absolute levels of lymphocyte subset populations in healthy men (supplement and placebo groups combined)*
(Mean values with their standard errors)

Cell type or surface marker † (10^9 cells/l)	Baseline (October/November)		Week 2 (November)		Week 14 (February)		Week 16 (February/March)		Week 18 (March/April)		Week 22 (April/May)		*P* (effect of time)	*P* (effect of time–supplement interaction)
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE		
CD19+	0·20[ac]	0·01	0·21[a]	0·01	0·17[b]	0·01	0·19[ab]	0·01	0·18[ab]	0·01	0·17[b]	0·01	0·000	0·777
n	37		37		38		38		38		37			
CD3+CD45RO+	0·79[bc]	0·04	0·79[c]	0·04	0·70[ab]	0·03	0·71[ab]	0·03	0·67[a]	0·03	0·71[ac]	0·03	0·000	0·161
n	35		37		38		37		38		38			
CD3+CD54+	0·50[b]	0·04	0·41[ab]	0·03	0·36[a]	0·03	0·40[ab]	0·04	0·42[ab]	0·04	0·49[b]	0·04	0·000	0·178
n	28		37		37		36		36		38			

CD, cluster of differentiation.
[abc]Mean values within a row with unlike superscript letters were significantly different from each other (analysed by paired *t* tests using Bonferroni's correction for multiple comparisons).
* For details of subjects and procedures, see Table 1 and p. 696.
† There was no alteration in the absolute levels of basophils, eosinophils, monocytes, neutrophils, CD3+, CD3-(CD16+56)+, CD3+CD4+, CD3+CD8+, CD3+HLA-DR, CD3+CD45RA+, CD3+CD25+, CD3+CD11a+.

with our findings that Zn supplementation (30 mg/d) had no effect on absolute numbers of circulating leucocytes in our younger healthy male subjects. No effect of Zn supplementation (400 mg/d for 60 d in three separate trials) on lymphocyte subsets has been observed in 150 elderly subjects after influenza vaccine (Provinciali *et al.* 1998). In our trial, a trend towards lower HLA-DR expression on T cells was seen after 14 weeks Zn supplementation (Table 3). Kelley *et al.* (1995) have shown that Cu depletion in healthy males lowers T cell HLA-DR expression. The present study did not include three colour FACS analysis. However, the design of future trials would be enhanced by the addition of a third marker to allow verification of the T cell subset (i.e. CD4+ or CD8+) being examined for the expression of the activation markers (interleukin-2 receptor and HLA-DR) and adhesion molecules (ICAM-1 and leucocyte function-associated antigen-1). Furthermore, the measurement of functional markers of immune status, for example, phagocytic activity of neutrophils and/or blastogenic response of cell types to T cells would further enhance our understanding of immune function.

Significant alterations in several lymphocyte subpopulations (absolute values) in both supplemented and placebo groups as a whole were observed over the study period. Lymphocyte subsets (as assessed by flow cytometry) altered over the time course of the trial were B cells, memory T cells, and T cell expression of the adhesion molecule CD54 (ICAM-1). B cell counts were significantly depressed over the winter months (November–January). Expression of CD45RO decreased from November to January and increased back towards baseline levels by the end of the trial in May. Expression of CD54 was reduced significantly over the winter months and had returned to baseline levels at completion of the trial. To support these findings, statistical analyses were also carried out on all cell subset data expressed as proportion (%) of cells within the lymphocyte lineage (data not shown). Significant temporal alterations were observed in the same three subsets (B cells, CD54 and CD45RO).

Several groups have observed significant seasonal differences in total number of peripheral blood leucocytes with peaks occurring in winter and troughs in summer (Reinberg *et al.* 1980; Gidlow *et al.* 1983; Reinberg & Smolensky, 1983). These findings are in contrast with results from the present study which observed reductions in circulating levels of lymphocyte subsets in winter. However, in agreement with our observations are experimental data that have demonstrated alterations in T cell subsets and B cell numbers (Abo & Kumagai, 1978; MacMurray *et al.* 1983) with reduction in winter *v.* autumn and summer. The seasonal alterations evident in a number of lymphocyte subsets (after statistical analyses) in the present trial were purely observational and unexpected. The changes were evident in both placebo and supplement groups and independent of supplement (Table 3) and a time–supplement interaction (Table 4). This observation raises an important issue in terms of devising longitudinal studies which run over changes in season and emphasises the need to differentiate between variability in results due to analytical procedures and subject variability over a period of time or a real host-related seasonal effect.

To our knowledge this is the first trial to examine the effect of Zn supplementation at the upper level (and NOAEL) for Zn on immune status. Calculation of the NOAEL for Zn is determined by dividing the lowest-observed-adverse-effect level of Zn of 60 mg/d by an uncertainty factor of 1·5 to give a NOAEL for Zn of 40 mg/d (Institute of Medicine, Food and Nutrition Board, 2001). There appears to be no adverse, or beneficial, effects of 14 weeks Zn supplementation (30 mg/d) on numbers of circulating leucocytes and flow cytometric analyses of lymphocyte subsets. Additionally, no effect of Zn supplementation on putative measures of Cu and Zn status was observed. These results indicate that doses of supplemental Zn up to 30 mg/d for 14 weeks appear non-hazardous and support evidence that the published NOAEL of Zn at 40 mg/d (30 mg/d supplement, 10 mg/d dietary intake) is safe for the majority of individuals. Independent of supplement, seasonal variations in circulating levels of lymphocyte subsets were observed. These changes could represent suppression of the immune system over the winter months with molecules important in acquired immunity (CD54, B cells and memory T cells) significantly depressed from November through to January. This could have implications for seasonal variations in the incidence of infectious diseases.

Acknowledgements

This work was funded by the Food Standards Agency (AN0553). We thank the Causeway Health and Social Services Trust Laboratories, Coleraine, Northern Ireland, UK for full blood profile analyses, Neil Dennison for his technical assistance, Professor Adrian Dunne for his statistical advice, Dr D. A. Hughes, Dr L. Harvey and Professor S. Fairweather-Tait for consultancy advice (Institute of Food Research, Norwich, UK) and Thomson and Joseph Ltd, Norwich, UK for supplying supplements.

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