BioMetals **13:** 123–133, 2000.

Influence of aluminium on the immune system – an experimental study on volunteers

Anna Gräske[1], Ann Thuvander[1], Anders Johannisson[2], Ingalill Gadhasson[1], Andrejs Schütz[3], Roger Festin[4] & Anders Wicklund Glynn[1,5,*]

[1]*National Food Administration, Box 622, SE-751 26 Uppsala, Sweden*
[2]*Department of Pathology, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Box 7028, SE-750 07 Uppsala, Sweden*
[3]*Department of Occupational and Environmental Medicine, University Hospital, SE-221 85 Lund, Sweden*
[4]*Dakopatts AB, Box 13, SE-125 21 Älvsjö, Sweden*
[5]*Department of Environmental Toxicology, Uppsala University, Norbyvägen 18A, SE-752 36 Uppsala, Sweden*
**Author for correspondence (Fax:018-171433;E-mail:glwi@slv.se)*

Received 10 February 2000; Accepted 17 February 2000

Key words: Al, human, lymphocytes, urine concentration

Abstract

The purpose of this study was to examine whether oral exposure to aluminum (Al) can affect the human immune system. Eighteen healthy volunteers (mean age 42, 28–57 yr) were divided into a test group (9 females, 4 males) and a referent group (3 females, 2 males). Over 6 weeks, the test subjects ingested 10 ml of antacid (aluminum hydroxide, 59 mg Al/ml) three times daily. Aluminum was analyzed in urine before and during the exposure period (ICP-MS). Blood samples were used for analysis of lymphocyte subpopulations, mitogen-induced lymphocyte proliferation and *in vitro* production and circulating plasma concentrations of immunoglobulin (Ig) A, IgG, IgM, interleukin (IL) -2 and IL-4. Urinary Al concentration in the test subjects was approximately 10- to 20-fold higher than in the referent group during exposure. This indicates that ingestion of an Al-containing antacid is associated with an Al absorption far above that originating from food and drinking water. In both referents and test subjects the lymphocyte subpopulations, lymphocyte proliferation and the *in vitro* Ig and IL production showed similar, time-dependent changes before as well as during the exposure period. No major differences were seen between the referent and test groups regarding the immune parameters, except for a slightly smaller CD8+CD45R0+ population (primed cytotoxic T-cells), in the exposed individuals as compared to the referents. The results also show that subjects on antacid therapy may constitute a suitable population for studying biological effects of high-dose oral exposure to Al.

Introduction

Aluminum is the third most common element in the earth's crust, comprising about 8% of its weight. The main sources of human exposure are food, drinking water and inhaled dust, but pharmaceuticals such as antacids and buffered analgesics also contribute to the exposure (Pennington & Jones 1988; Sjögren *et al*. 1990; Flaten *et al*. 1996). The intestinal absorption of Al depends on the pH in the gastro-intestinal tract and it is also strongly influenced by complex-binding ligands in the food and drinking water (Rodger *et al*. 1991). Aluminum is relatively non-toxic to healthy individuals due to rapid excretion after absorption. However, in patients with renal disease, exposure to Al in dialysis water has been found to cause dementia, anemia and osteomalacia (Wills & Savory 1989).

Due to the stimulatory effect of Al on certain parts of the immune system, Al salts have been used as adjuvant in vaccines for decades (Nicklas 1992). In

animal studies, Al has been shown to modulate the immune system. Ramanathan *et al*. (1979) found that Al-hydroxide could activate the complement system in guinea pigs. Moreover, exposure of male Sprague-Dawley rats to 500 mg Al/l drinking water caused a slight stimulation of certain immune parameters (Wicklund Glynn *et al*. 1999). Other animal studies (Yoshida *et al*. 1989; Golub *et al*. 1993), on the other hand, indicate that Al may have an immunosuppressive effect when it is given in high doses. It has also been suggested that Al may cause suppression of cell-mediated immune mechanisms in patients with kidney dysfunction (Nordal *et al*. 1988).

Little is known about the effects of Al in orally exposed healthy humans. Therefore, this study aimed to examine if high-dose oral exposure to Al affects the human immune system in healthy volunteers. An Al-containing antacid was used as a source of Al exposure. The daily Al intake during normal use of this antacid was estimated to be approximately 100- to 200-fold higher than the average daily intake from food and drinking water.

Materials and methods

Experimental design and exposure

Twenty healthy volunteers from the city of Uppsala in Sweden gave their informed consent to take part in the study, which was approved by the Ethics Committee of the Medical Faculty at Uppsala University. Only healthy subjects who had not previously been taking antacids and who had not been vaccinated during the 3 months prior to the study were allowed to participate. Individuals with iron deficiency or individuals using pharmaceutics that interact with antacids were excluded. Due to iron deficiency, one of the subjects was excluded before the start of the study and a second volunteer was not able to complete the study because of side effects (constipation problems) due to antacid intake. Infections in the subjects were recorded during the study. However, only minor colds in 4 of the subjects (2 reference and 2 exposed subjects) were reported. Due to the mildness of the infections it was decided to keep these subjects in the study.

The 18 subjects included in this open study were randomly assigned into two groups. The Al-exposed group consisted of 13 subjects, 9 women (mean age 42 years, range 28–57 years) and 4 men (mean age 42 years, range 32–51 years). The 13 subjects ingested Al hydroxide in the form of an antacid suspension, (Novaluzid, Hässle, Mölndal, Sweden) for six weeks. Three daily doses of 10 ml antacid mixture (Al content 588 mg/10 ml, about 1.8 g Al/day) were given. The remaining 5 subjects, 3 women (mean age 44 years, range 35-53 years) and 2 men (mean age 40 years, range 35–45 years) were used as referents and did not take any antacids/Al-containing drugs during the study.

Sampling of blood and urine

Blood samples were taken for measurements of mitogen-induced proliferation, lymphocyte subpopulations and immunoglobulins and interleukins in plasma and supernatants from lymphocyte cultures. Iron status was monitored during the whole study period. All subjects had an iron status within the normal range at all samplings. Before the start of the Al exposure, two base-line blood samples were taken four weeks apart and the immune parameters described above, as well as iron status, were measured. The second sampling was performed on the day before the start of the exposure. During the 6-week exposure to Al, three blood samples were drawn from the subjects for analysis of immune parameters and iron status. The blood samples were taken two weeks apart, starting two weeks after the exposure. The third blood sample was drawn on the last day of exposure. At each sampling, 36 ml of venous blood was collected from each subject. Sodium heparin vaccutainer tubes (Becton Dickinson, England) were used to collect 28 ml of blood for measurement of the immunological parameters. For analysis of iron status 8 ml of blood was collected in SST gel vaccutainer tubes (Becton Dickinson, England).

The body burden of Al before and during antacid exposure was determined by measurements of urinary Al excretion. Morning urine (20 ml/ind) was collected in Al-free plastic tubes (Labora, Sollentuna, Sweden) after overnight fasting. Sampling was performed on the day before the onset of the experiment to obtain a baseline determination and then 3 times during the exposure period in conjunction with the blood sampling. The urine samples were acidified by adding 0.5 ml subboiled distilled nitric acid to 10 ml of urine and stored frozen until analysis. Special precautions were taken to prevent extraneous Al contamination.

Analysis of urine creatinine, aluminum and iron status

To estimate Al excretion, urine creatinine levels were determined at the University Hospital, Uppsala. Urine creatinine was measured by a routine laboratory analytical technique. Aluminum in urine was analyzed at the Departure of Occupational and Environmental Medicine, University Hospital, Lund, by inductively coupled plasma mass spectrometry (ICP-MS). A low-resolution spectrometer (VG PQ2+, Fisons Elemental, Winsford, Cheshire, UK) equipped with an autosampler (Gilson 222, Gilson, Villiers, France) was used. The samples (0.50 ml urine) were diluted 10-fold with a solution containing EDTA (0.5 g/l), Triton-X100 (0.5 g/l) and ammonia (5 ml/l) in Millipore water. Gallium and indium were added as internal standards. The isotopes (^{27}Al, ^{69}Ga, ^{71}Ga and ^{115}In) were monitored in pulse counting, peak jumping mode (3 points per peak). Each sample was prepared in duplicate and the mean of both determinations was used. Spiked urine was used for method calibration. The detection limit (3× SD for reagent blanks) was 0.07 μmol/l.

Blood samples were sent to the Center for Laboratory Medicine, University Hospital, Uppsala, where iron levels in blood, including serum iron, total iron binding capacity (TIBC), transferrin and ferritin were determined by standard laboratory methods.

Preparation of mononuclear cells

Blood from the subjects was diluted with an equal volume of PBS (Phosphate Buffered Saline) and layered on Ficoll-Paque (Pharmacia, Uppsala, Sweden). After centrifugation (400 g) of the samples at room temperature for 30 min the cells, at the interface, were collected. Blood lymphocytes were washed twice in cold PBS and once in cell complete medium, (RPMI 1640) with 10% heat-inactivated Fetal Bovine Serum (FBS, Flow Laboratories, Ltd), 5000 μg/ml streptomycin, 5000 IU/ml penicillin, 2 mmol L-glutamine, 0.05 mmol mercaptoethanol and 20 mmol Hepes (complete medium). The cells were then resuspended in complete medium and counted.

Lymphocyte proliferation assay

The lymphocyte cell concentration was adjusted to 2.7×10^6 cells/ml complete medium. The cell suspension was seeded at 75 μl per well in round-bottomed microtiter plates (Labassco, Stockholm, Sweden). The mitogens phytohaemagglutinin (PHA, Chemicon AB, Malmö, Sweden) and pokeweed mitogen (PW, Sigma, St Louis, MO) were diluted in PBS and added together with 100 μl of complete medium in 25 μl volumes to provide the required final concentrations in the cultures of 10 μg PHA/ml and 2 μg PW/ml, respectively. All assays were performed in triplicate. The lymphocytes were cultured for 72 h at 37 °C in an atmosphere of 5% CO_2 in air. After 48 h the lymphocyte cultures were supplemented with 25 μl complete medium containing 0.5 μCi [methyl-3H]thymidine (Amersham, UK) and the cells were cultured for another 24 h. Thereafter, the lymphocytes were harvested and the cellular uptake of radioactivity per culture was measured in a betaplate counter (LKB Wallac, Finland). Results are expressed as mean cpm values ±SD for each study group.

Analysis of lymphocyte subpopulations

For analysis of lymphocyte subpopulations, 1×10^5 cells were stained in 50 μl complete medium containing 1 μl fluorescein isothiocyanate (FITC), 1 μl R-phycoerythrin (RPE) and 1 μl R-phycoerythrin-Cy5 (RPE-Cy5) conjugated antibodies. The cells were stained with combinations of different conjugated antibodies in 9 different tubes per subject and sampling.

The following subpopulations were evaluated:

CD19+ cells (B-lymphocytes except plasma cells), CD19+CD23+ cells (activated B-lymphocytes), CD5+ cells (T- and some B lymphocytes), CD3+ cells, (T-lymphocytes), CD4+ cells (T-helper cells), CD8+ cells (T-cytotoxic cells), CD4+HLA-DR+ cells (activated T-helper cells), CD8+HLA-DR+ cells (activated T-cytotoxic cells), CD4+CD45RA+ cells (naive or virgin T-helper cells), CD8+ CD45RA+ cells (naive or virgin T-cytotoxic cells), CD4+ CD45R0+ cells (primed T- helper cells), CD8+ CD45R0+ cells (primed T- cytotoxic cells), CD4+ CD45RA^{int} R0^{int} cells and CD8+ CD45RA^{int}R0^{int} cells (intermediate forms), CD56+ cells (NK-cells), CD16+ cells (NK-cells and granulocytes, but also to a lesser extent macrophages and monocytes).

Cells stained with isotype control antibodies were used as negative controls. All antibodies were from DAKO A/S, Denmark, except for the HLA-DR antibodies that were from Becton Dickinson, San Jose, USA. Staining proceeded in darkness for 60 min on ice. The cells were then washed twice with cold PBS and resuspended in complete medium. They were kept dark on ice until analysis.

The flow cytometry analysis was conducted with a FACStar^{PLUS}(Becton Dickinson Immunocytometry Systems) flow cytometer. Spectra-Physics 2016 argon laser (Spectra-Physics, Mountain View, CA, USA) tuned to 488 nm and operating at 200 mW was used as a light source. SSC-H from the 488 m laser was collected through a 490/20 m bandpass filter. FITC fluorescence (FL1-H) was collected through a 530/30 m bandpass filter; RPE fluorescence (FL2-H) through a 580/30 m bandpass filter; and RPECy5 fluorescence (FL3-H) through a 660/20 m bandpass filter. The light scatter parameters were collected using linear amplification; the antibody fluorescence parameters were collected using logarithmic amplification. A five-parameter (FSC, SSC, FL1, FL2, FL3) listmode file of 50 000 events was collected from each sample. Data were collected with a FACStation running CELLQUEST software version 1.2.2. Cells inside a lymphocyte gate were further analyzed for expression of surface markers.

In vitro *Ig production*

The blood lymphocyte cell concentration was adjusted to 1.3×10^6 cells/ml. In a flat-bottomed 24 well plate (Nunc, Roskilde, Denmark), 750 μl cell suspension was seeded per well and 250 μl volumes of PW and 1000 μl of complete medium were added to provide the final concentrations in the cultures of 2 μg PW/ml. All assays were performed in duplicate. The lymphocytes were cultured for 7 days at 37 °C in an atmosphere of 5% CO_2 in air. After 3 days, 1 ml of the supernatant was removed from each of the cultures and replaced with an equal volume of fresh complete medium. After 7 days, aliquots of the supernatants were carefully removed from the cultures and tested for immunoglobulins in an ELISA assay.

In vitro *interleukin production*

The blood lymphocyte cell concentration was adjusted to 2.7×10^6 cells/ml. In a flat-bottomed 24 well plate (Nunc, Roskilde, Denmark), 750 μl cell suspension was seeded per well and 250 μl volumes of concanavalin A (Con A) and 1000 μl of complete medium were added to provide the final concentration in the cultures of 5 μg Con A/ml. All assays were performed in duplicate. The lymphocytes were cultured for 48 h at 37 °C in an atmosphere of 5% CO_2 in air, aliquots of the supernatants were then carefully removed from the cultures and tested for interleukins in an ELISA assay.

ELISA for quantification of IgA, IgG and IgM in plasma and supernatants

To quantify the production and the circulating IgA, IgG and IgM concentrations, a standard indirect enzyme-linked immunosorbent assay (ELISA) was used. Microtiter plates (Nunc, Roskilde, Denmark) were coated over night at 4 °C with polyvalent goat anti-human immunoglobulins (Sigma, St Louis, MO) diluted 1:5000 in coating buffer (0.05 m carbonate-bicarbonate buffer pH 9.6). All washings and dilutions of plasma and supernatants of the peroxidase-labelled goat anti-human IgA, IgM and IgG (Sigma, St Louis, MO) were made with PBS containing 0.05% Tween 20. All incubations (100 μl volumes) were performed at 37 °C for 120 min. Plasma and supernatants samples were diluted and tested in two-step titrations. The peroxidase-conjugate was diluted 1:5000 for the plasma samples and 1:1000 for the supernatant samples. The enzyme reaction was visualized using a substrate-solution containing tetramethyl-benzidine (0.1 mg/ml) and H_2O_2 (0.006%) in 0.1 M acetate, pH 6.0 (EC Diagnostics, Uppsala, Sweden). The reaction was stopped after 10 min by addition of 50 μl 1M H_2SO_4 to each well and the absorbance was measured at 450 nm with a Microplate Bio-kinetics reader EL312e (Bio-Tek Instruments, Kebo Lab, Stockholm, Sweden). The concentrations of IgA, IgG and IgM were calculated from two-step titrations of standard preparations of human IgA, IgG and IgM (Chemicon AB, Malmö, Sweden) which was included on each microtiter plate.

ELISA for quantification of IL-2 and IL-4 in plasma and supernatants

To quantify the concentrations of the interleukins IL-2 and IL-4, an ELISA Development Kit (R & D Systems, Minneapolis, USA) was used. Concentrations of IL-2 and IL-4 in plasma and in supernatants were calculated from three-step titrations of standard preparations of human IL-2 and IL-4, which were included on each microtiter plate. The following modifications were made to the kit: Samples were incubated for 2 h at 37 °C instead of 1 h at room temperature, the incubation with detection antibody was performed at 37 °C instead of room temperature and peroxidase-conjugate was incubated for 1 h instead of 20 min.

Statistical analysis

Differences between the exposed group and the referents prior to the study (baseline determination) were evaluated using the Mann–Whitney unpaired U-test. Results from the three samplings during exposure were evaluated using repeated measures ANOVAs. These tests were used in order to investigate whether the curves, based on mean values from referents and exposed subjects, were parallel or not. When the curves were not parallel, the two groups were compared using the Mann–Whitney unpaired U-test. When the curves were parallel, repeated measures ANOVAs were used to evaluate differences between the two groups during the exposure period. Repeated measures ANOVAs were also used to evaluate differences over time. Differences were considered to be statistically significant at P less than 0.05.

Results

Excretion of aluminum in urine

No significant differences in Al excretion were seen between the two groups prior to the Al exposure (Figure 1). The urinary excretion was significantly higher in the exposed group after 2 weeks of exposure. There was no significant change in Al excretion over time in the referents.

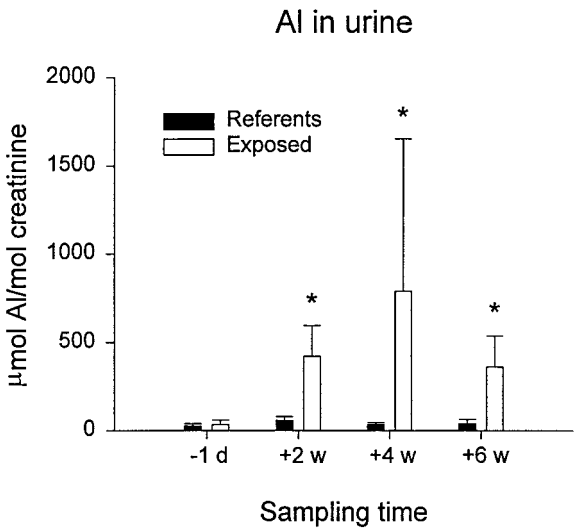

Fig. 1. Urinary excretion of Al in 5 reference and 13 Al-exposed subjects expressed as mean μmol Al/mol creatinine $\pm$ SD one day before and 2, 4, and 6 weeks after the start of exposure. A significant change over time during the study period was seen for the exposed individuals (Repeated measures ANOVAS, $p < 0.05$). *Significantly different from corresponding reference value when tested statistically (Repeated measures ANOVAS, $p < 0.05$).

B-lymphocyte subpopulations

A significant decrease in the proportion of CD5+ cells was observed in the exposed group on the second sampling during antacid exposure (Table 1). The proportion of CD19+CD23+ cells differed between the groups at the first baseline sampling, but no such differences were seen on any other sampling occasion. No significant differences were seen in the proportion of CD19+ cells between referents and exposed subjects.

T-lymphocyte subpopulations

The proportion of CD3+ cells did not differ between referents and exposed subjects (Table 1), nor were there any significant differences between the two groups in the proportions of CD4+ cells or CD8+ cells. The percentage of CD8+CD45R0+ cells was significantly lower in the exposed group as compared to the referents on all sampling occasions during the period of antacid ingestion, but not at the base-line samplings, indicating an exposure-related effect (Figure 2). The proportion of CD8+CD45RA+, CD8+CD45RA^{int}R0^{int}and CD8+HLA-DR+ cells were similar in referent and exposed subjects.

No differences between referents and exposed individuals were seen in the proportion of activation related markers on CD4+ cells.

NK-cells

Two NK-cell markers were studied, CD56 and CD16 (Table 1). Neither the population of CD16+ cells nor the population of CD56+ cells differed between the groups.

Lymphocyte proliferation

No significant differences were found between the proliferation of lymphocytes from referents and lymphocytes from exposed subjects (Figure 3).

Table 1. Flow cytometric analysis of lymphocyte subpopulations expressed as percent of lymphocytes for 5 referents and 13 Al-exposed subjects[a].

Sampling[b] occasion	CD5+	CD19+	CD19+ CD23+	CD3+	CD8+	CD8+ CD45 RA+	CD8+ CD45 RA^{Int} $R0^{Int}$	CD8+ CD45 R0+	CD8+ HLA -DR+	CD4+	CD4+ CD45 RA+	CD4+ CD45 RA^{Int} RO^{Int}	CD4+ CD45 R0+	CD4+ HLA -DR+	CD16+	CD56+
−4 w																
Referents	64 ± 9	10 ± 4	3 ± 1	59 ± 6	18 ± 5	48 ± 10	27 ± 8	18 ± 6	14 ± 8	48 ± 10	33 ± 6	18 ± 4	41 ± 11	5 ± 2	11 ± 4	0.61 ± 0.57
Exposed	57 ± 7	16 ± 7	6 ± 2[c]	55 ± 8	16 ± 6	49 ± 11	28 ± 12	15 ± 6	18 ± 10	45 ± 8	31 ± 8	19 ± 10	43 ± 13	6 ± 2	12 ± 4	0.43 ± 0.26
−1 d																
Referents	56 ± 9	9 ± 3	5 ± 3	42 ± 8	19 ± 4	46 ± 13	42 ± 11	7 ± 2	8 ± 2	39 ± 9	33 ± 10	24 ± 4	36 ± 13	6 ± 2	18 ± 7	0.22 ± 0.07
Exposed	49 ± 7	14 ± 5	9 ± 3	38 ± 5	15 ± 5	50 ± 13	38 ± 12	5 ± 2	13 ± 8	36 ± 7	32 ± 9	24 ± 12	36 ± 9	5 ± 2	16 ± 8	0.25 ± 0.32
+2 w																
Referents	66 ± 7	9 ± 4	4 ± 2	65 ± 9	16 ± 6	50 ± 9	34 ± 7	9 ± 3	3 ± 1	51 ± 7	40 ± 9	18 ± 3	36 ± 9	3 ± 1	12 ± 7	0.28 ± 0.06
Exposed	57 ± 7	13 ± 5	7 ± 3	60 ± 6	14 ± 5	56 ± 8	30 ± 8	6 ± 2[d]	5 ± 3	47 ± 5	40 ± 9	16 ± 5	39 ± 8	3 ± 1	14 ± 6	0.28 ± 0.27
+4 w																
Reference	58 ± 7	12 ± 5	5 ± 3	58 ± 8	17 ± 6	62 ± 9	19 ± 5	12 ± 4	2 ± 1	43 ± 7	48 ± 9	18 ± 4	30 ± 9	3 ± 1	14 ± 6	0.14 ± 0.04
Exposed	46 ± 9[c]	18 ± 7	8 ± 3	47 ± 8	13 ± 4	65 ± 11	22 ± 11	8 ± 3[d]	3 ± 2	34 ± 9	46 ± 9	20 ± 10	30 ± 8	3 ± 1	18 ± 8	0.23 ± 0.41
+6 w																
Referents	48 ± 5	8 ± 4	3 ± 2	60 ± 6	19 ± 7	66 ± 4	21 ± 3	9 ± 2	6 ± 1	40 ± 5	47 ± 8	20 ± 2	26 ± 8	4 ± 1	16 ± 8	0.09 ± 0.06
Exposed	50 ± 12	14 ± 6	6 ± 3	61 ± 14	17 ± 6	67 ± 13	22 ± 11	6 ± 3[d]	8 ± 4	42 ± 13	43 ± 9	21 ± 12	30 ± 8	4 ± 1	18 ± 11	0.08 ± 0.10

Values are expressed as mean ± SD of 5 referents and 13 Al-exposed subjects.
[a] All parameters changed significantly during the study period (repeated measures ANOVAS, $p < 0.05$).
[b] Four weeks and one day before and 2, 4 and 6 weeks after the start of exposure.
[c] Significantly different from the referents when tested statistically (Mann–Whitney unpaired U-test, $p < 0.05$).
[d] Significantly different from the referents when tested statistically (repeated measures ANOVAS, $p < 0.05$).

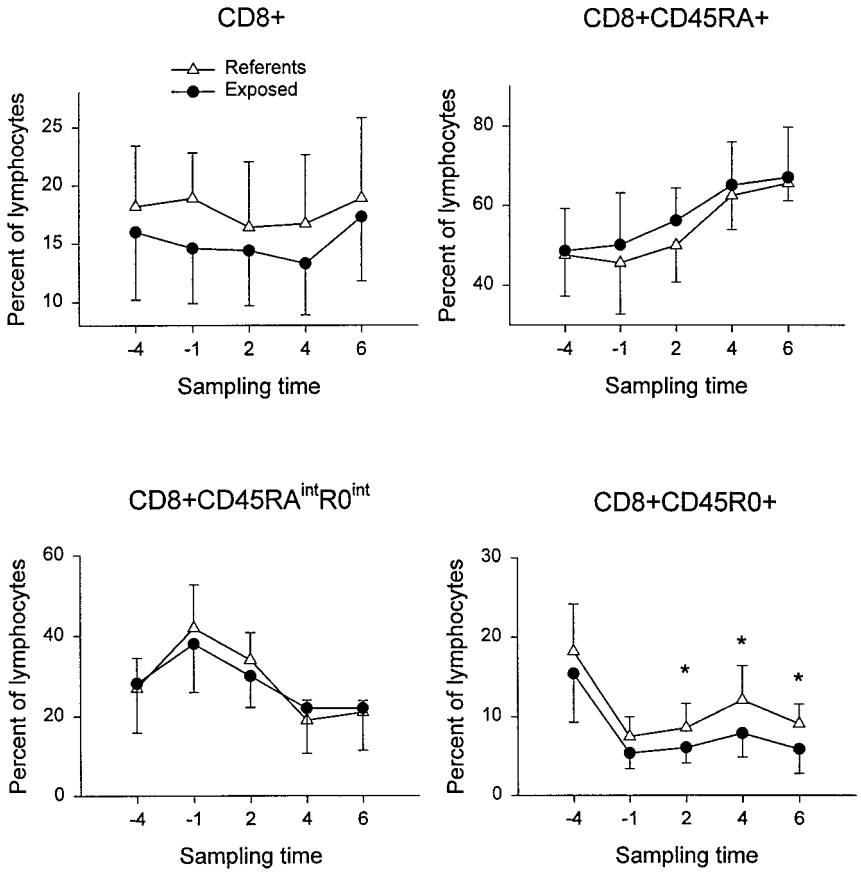

Fig. 2. Flow cytometric analysis of CD8+ T-cell subpopulations. Results are expressed as mean values ± SD four weeks and one day before and 2, 4, and 6 weeks after the start of exposure for 5 reference and 13 exposed subjects. A significant change over time during the study period was seen in all parameters in both groups (Repeated measures ANOVAS, $p < 0.05$). *Significantly different from corresponding reference value when tested statistically (Repeated measures ANOVAS, $p < 0.05$).

In vitro *production and plasma concentrations of immunoglobulins and interleukins*

The results from the *in vitro* Ig production assay showed considerable variations between individuals, but no exposure-related differences were seen between the groups (results not shown). None of the plasma concentrations of IgA, IgG nor IgM differed between referents and exposed subjects (Table 2).

The *in vitro* production of IL-2 and IL-4 was not affected by the Al exposure (Table 3). There was a significant difference in plasma concentration of IL-4 between referents and exposed subjects at the first baseline sampling and in the IL-4 production at the second baseline sampling, but no difference was seen on any other sampling occasion.

Variation over time

All B- and T-lymphocyte subpopulations, both in exposed and referent subjects, showed significant differences over time during the study period (Table 1, Figure 2). Furthermore, in the lymphocyte proliferation assay (Figure 3), and in the IgA, IgG, IgM, IL-2 and IL-4 production assays, significant differences over time were observed during the study (Table 3). No variation over time was found in immunoglobulin and interleukin concentrations in plasma.

Discussion

The purpose of this study was to evaluate the effects of oral Al exposure on some immunological endpoints. Although the immunostimulatory effect of Al when used as an adjuvant in vaccines is well known, no

Table 2. IgA, IgG, and IgM concentrations in plasma from 5 referents and 13 Al- exposed subjects.

Sampling occasion[a]	IgA (mg/ml)	IgG (mg/ml)	IgM (mg/ml)	
−4 w	Referents	7.1 ± 2.7	7.2 ± 0.7	0.3 ± 0.2
	Exposed	13 ± 19	7.9 ± 1.5	0.4 ± 0.2
−1 d	Referents	7.1 ± 1.3	6.8 ± 1.1	0.3 ± 0.2
	Exposed	8.2 ± 3.7	7.2 ± 1.9	0.3 ± 0.1
+2 w	Referents	8.4 ± 4.1	5.8 ± 2.0	0.3 ± 0.2
	Exposed	7.9 ± 3.3	7.0 ± 1.8	0.3 ± 0.1
+4 w	Referents	8.7 ± 5.0	6.4 ± 1.0	0.3 ± 0.2
	Exposed	7.6 ± 1.9	7.0 ± 1.5	0.3 ± 0.1
+6 w	Referents	5.5 ± 1.1	5.3 ± 0.9	0.2 ± 0.1
	Exposed	7.3 ± 2.3	6.8 ± 1.5	0.3 ± 0.1

Values are expressed as mean ± SD of 5 referents and 13 Al-exposed subjects.
[a]Four weeks and one day before and 2, 4 and 6 weeks after the start of exposure.

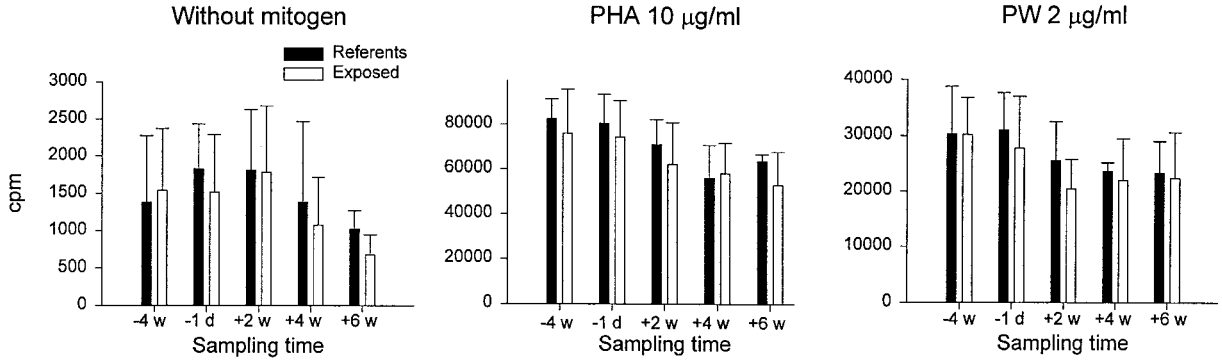

Fig. 3. Lymphocyte proliferation in cell cultures without mitogen, and proliferation of PHA (10 μg/ml) and PW (2 μg/ml) -stimulated cells from reference and Al-exposed subjects. Cultures without mitogen were always under 2000 cpm. The cells were cultured for 48 h before addition of [^{3}H]thymidine and were then cultured for another 24 hours before harvest. Results are expressed as mean cpm values four weeks and one day before and 2, 4, and 6 weeks after the start of exposure for 5 reference and 13 exposed subjects (3 wells per individual). Bars indicate standard deviation. A significant change over time during the study period was seen in all parameters in both groups (Repeated measures ANOVAS, $p < 0.05$).

study has focused on the modulatory effects of Al on the human immune system after oral exposure. It has been indicated that Al may cause both immunosuppressive and immunostimulatory effects in experimental animals (Yoshida *et al*. 1989; Golub *et al*. 1993; Wicklund Glynn *et al*. 1999).

Our hypothesis was that antacid therapy would result in a substantially increased intestinal absorption of Al, which would be reflected in an increased urinary excretion. For average food consumers it can be estimated that the intake of Al from food is approximately 100- to 200-fold lower than the intake of Al from antacids in this study.

The 10- to 20-fold increase in urinary Al in the individuals during antacid therapy confirms our hypothesis and shows that the subjects absorbed Al from the antacid. This finding agrees with earlier results demonstrating elevated concentrations of Al in urine and blood after ingestion of Al-containing antacids (Slanina *et al*. 1986; Weberg & Berstad 1986; Priest *et al*. 1996). Even from single doses of antacids it has been shown that measurable quantities of Al are absorbed (Weberg & Berstad 1986).

Based on the few animal studies reported, we predicted that the high-dose Al exposure might cause a stimulatory effect on the immune system. However, although several immune parameters were evalu-

Table 3. IL-2 and IL-4 concentrations in plasma and supernatants from concanavalin A stimulated cultures from 5 referents and 13 Al-exposed subjects.

Sampling occasion[a]		Plasma (pg/ml)		Supernatants (pg/ml)	
		IL-2	IL-4	IL-2[b]	IL-4[b]
−4 w	Referents	1.1 ± 0.8	0.05 ± 0.09	31 ± 12	8.1 ± 4.4
	Exposed	4.6 ± 4.8	2.1 ± 2.2[c]	21 ± 16	3.3 ± 3.4
−1 d	Referents	4.9 ± 4.6	2.0 ± 1.4	123 ± 132	15 ± 5.0
	Exposed	12.1 ± 23	6.0 ± 13	71 ± 41	7.2 ± 4.2[c]
+2 w	Referents	4.2 ± 3.1	2.5 ± 2.2	18 ± 15	9.4 ± 5.8
	Exposed	6.7 ± 15	2.2 ± 3.5	18 ± 9.8	6.0 ± 3.7
+4 w	Referents	2.1 ± 2.0	2.4 ± 1.8	116 ± 120	9.6 ± 3.0
	Exposed	7.4 ± 15	5.3 ± 11	114 ± 87	6.4 ± 3.5
+6 w	Referents	3.3 ± 1.1	2.6 ± 2.5	78 ± 72	3.1 ± 2.6
	Exposed	6.1 ± 11	4.2 ± 7.7	56 ± 44	3.5 ± 2.5

Values are expressed as mean ± SD of 5 referents and 13 Al-exposed subjects.
[a]Four weeks and one day before and 2, 4 and 6 weeks after the start of exposure.
[b]Significant change over time during the study period (Repeated measures ANOVAS, $p < 0.05$).
[c]Significantly different from the referents when tested statistically (Mann–Whitney unpaired U-test, $p < 0.05$).

ated in the study, almost no exposure-related effects were seen. This indicates that Al is not a potent immunomodulator in humans at these levels of oral exposure. The only consistent difference seen between the referents and the exposed group was a decrease in the CD8+CD45R0+ population (primed CD8+ cells) in the exposed group on the three sampling occasions during antacid therapy. No such difference was seen between the groups at the two baseline samplings prior to the exposure, indicating that it was not a random finding. The proportions of CD4+ subpopulations, including CD4+CD45R0+ cells, were not affected, suggesting a specific effect of Al on primed cytotoxic T-cells. Effects on cytotoxic T-cells in relation to Al exposure have, to our knowledge, not previously been studied. Our finding may imply a reduction in cytotoxic T-cell activity due to Al exposure. The CD8+ subpopulation is regarded primarily to consist of cytotoxic cells. However, it has also been suggested that CD8+ cells play an active role in the regulation of the immune response (Kemeny *et al*. 1994), where they may have a down-regulatory function (Kemeny *et al*. 1994; Lombardi *et al*. 1994; Chou *et al*. 1996). A relative reduction in cells with immuno-suppressive properties might result in immunostimulation. Thus, this finding could be in line with the stimulatory effects seen in some animal studies after exposure to Al (Ramanathan *et al*. 1979; Yoshida *et al*. 1989; Wicklund Glynn *et al*. 1999). It is also in accordance with the known stimulatory effect of Al when used as an adjuvant (Nicklas 1992).

Measurements of lymphocyte subpopulations are often used to reveal changes in the immune system due to low-dose exposure to toxic substances (Van Loveren *et al*. 1995; Weisglas-Kuperus *et al*. 1995), but the effects of Al on lymphocyte subpopulations have, so far, been investigated in only one animal study. Exposure of Swiss Webster mice to 1000 mg Al lactate/kg diet, from conception to 6 months of age, caused a depressed cytokine production and a decrease in the proportion of CD4+ cells (Golub *et al*. 1993). The CD8+ subpopulation was not affected by Al exposure. The proportion of CD8+CD45R0+ cells was not investigated in the study by Golub *et al*. (1993). Taken together the results of the mice study indicated an immunosuppression, which is contrary to our results. The Al intake of the Swiss Webster mice was, however, about 4-fold higher than in the exposed human subjects in our study. Furthermore, the volunteers in our study ingested Al for a much shorter period. This could at least partially explain why we did not see any effects on the CD4+ population. Thus, it seems likely that Al may induce both stimulatory and suppressive effects on the immune system, depending on dose, route of administration and duration of exposure.

Although the Al exposure slightly affected lymphocyte subpopulations, no effects were seen on the other immune parameters measured in our study. Previous studies on the effect of Al on mitosis in human

short-term whole blood cultures have indicated that lower concentrations (2 μm $AlCl_3$, a concentration close to that found in normal serum) enhance mitosis, while higher concentrations (2.0–4.0 mM $AlCl_3$) inhibit mitosis (Yao *et al*. 1994). In contrast to these findings, the mitogen-induced proliferation of human peripheral lymphocytes in the present study was not affected by Al exposure.

All lymphocyte subpopulations showed a statistically significant variation over time both in exposed subjects and referents. Similarly, the results from the lymphocyte proliferation test and the *in vitro* production of IgA, IgG, IgM, IL-2 and IL-4 differed significantly between the sampling occasions. Trends over time in immunological parameters are not very well established in humans. However, there are some studies indicating seasonal rhythms in some human immune variables, such as the transferrin receptor (TfR), which is expressed on activated lymphocytes after antigenic or mitogen-induced stimulation (Maes *et al*. 1997). Moreover, in field studies of birds and mammals, the immune function is known to be compromised during the winter (Nelson *et al*. 1997). Due to the time trends found in our study, it was not possible to use the individuals as their own referents, which would have been the optimal study approach.

In conclusion, the results from the present study show that exposure of healthy humans to Al-containing antacids is a suitable model for studies of biological effects of high-dose oral Al exposure.The absorption of Al, and thus the body burden, increases dramatically during antacid exposure. Although the Al exposure in this study was considerably higher than that from food, there were no clear signs of immunomodulation in the parameters measured, except for a slight suppression of primed CD8+ cells. It seems plausible to conclude that the immunotoxic potential of Al from food is limited, even though only a rather small number of subjects were exposed during a relatively short time in the present study.

Acknowledgements

We thank Anders Ekholm, Irma Häggbom and Ebba Hallberg for their excellent technical assistance. The flow cytometer used in this study was financed by a grant from the ‘Knut & Alice Wallenberg foundation’.

References

Chou KY, Chan M, Bias WB. 1996 Differential expression of the down-regulatory function of CD8 cells in trichsanthin-induced immunosuppression and its genetic control in humans. *Eur J Immunogen* **23**, 29–40.

Flaten TP, Alfrey AC, Birhall JD, Savory J, Yokel RA. 1996 Status and future concerns of clinical and environmental aluminum toxicology. *J Toxicol Environ Health* **48**, 527–541.

Golub MS, Takeuchi PT, Gershwin ME, Yoshida SH. 1993 Influence of dietary aluminum on cytokine production by mitogen-stimulated spleen cells from Swiss Webster mice.*Immunopharmcol Immunotoxicol* **15**, 605–619.

Kemeny DM, Noble A, Holmes BJ, Diaz-Sanchez D. 1994 Immune regulation: a new role for the CD8+ T cell. *Immunology Today* **15**, 107–110.

Lombardi G, Sidhu S, Batchelor R, Lechler R. 1994 Anergic T cells as suppressor cells in vitro. *Science* **264**, 1587–1589.

Maes M, Bosmans E, Scharpé S *et al*. 1997 Components of biological variation in serum soluble transferrin receptor: relationships to serum iron, transferrin and ferritin concentrations, and immune and haematological variables. *Scand J Clin Lab Invest* **57**,31–41.

Nelson RJ, Demas GE. 1997 Role of melatonin in mediating seasonal energetic and immunologic adaptations. *Brain Res Bull* **44**, 423–430.

Nicklas W. 1992 Aluminum salts. *Res Immunol* **143**, 489–494.

Nordal KP, Dahl E, Alrechtsen D *et al.* 1988 Aluminium accumulation and immunosuppressive effects in recipients of kidney transplants. *Br Med J* **297**, 1581–1582.

Pennington JAT, Jones JW. 1988 Dietary intake of aluminum. In: Gitelman HJ, eds. *Aluminum in Health, a Critical Review*. New York: Marcel Dekker, pp. 67–98.

Priest ND, Talbot RJ, Austin JG *et al*. 1996 The bioavailability of ^{26}Al-labelled aluminium citrate and aluminium hydroxide in volunteers. *BioMetals* **9**, 221–228.

Ramanathan VD, Badenoch-Jones P, Turk JL. 1979 Complement activation by aluminium and zirconium compounds. *Immunology* **37**, 881–888.

Rodger RSC, Muralikrishna GS, Halls DJ *et al.* 1991 Ranitidine suppresses aluminium absorption in man. *Clin Science* **80**, 505–508.

Sjögren B, Gustavsson P, Hogstedt C. 1990 Neuropsychiatric symptoms among welders exposed to neurotoxic metals. *Br J Ind Med* **47**, 704–707.

Slanina P, Frech W, Ekström LG, Lööf L, Slorach S, Cedergren A. 1986 Dietary citric acid enhances absorption of aluminium in antacids. *Clin Chem* **32**,539–541.

Van Loveren H, Steerenberg PA, Vos JG. 1995 Early detection of immunotoxicity: from animal studies to human biomonitoring. *Toxicol Lett* **77**, 73–80.

Weberg R, Berstad A. 1986 Gastrointestinal absorbtion of aluminium from single doses of aluminium-containing antacids in man. *Eur J Clin Invest* **16**, 428–432.

Weisglas-Kuperus N, Sas TCJ, Koopman-Esseboom C *et al*. 1995 Immunologic effects of background prenatal and postnatal exposure to dioxins and polychlorinated biphenyls in Dutch infants. *Pediatr Res* **38**, 404–410.

Wicklund Glynn A, Thuvander A, Sundström B, Sparen A, Danielsson LG, Jorhem L. 1999 Does aluminium stimulate the immune system in male rats after oral exposure? *Food Add Contam* **16**, 129–135.

Wills MR, Savory J. 1989 Aluminium and chronic renal failure: sources, absorption, transport and toxicity. *Crit Rev Clin Sci* **27**, 59–107.

Yao XL, Jenkins ED, Wisniewski HM.1994 Effects of Aluminum Chloride on mitogenesis, mitosis, and cell cycle in human short-term whole blood cultures: Lower concentrations enhance mitosis. *J Cell Biochem* **54**, 473–477.

Yoshida S, Gershwin ME, Keen CL, Donald JM, Golub MS. 1989 The influence of aluminium on resistance to Listeria monocytogenes in Swiss-Webster mice. *Int Arch Allergy Appl Immunol* **89**, 404–409.