



Smokeless tobacco or nicotine replacement therapy has no effect on serum immunoglobulin levels[☆]

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Summary Background: Tobacco smokers have lower serum levels of immunoglobulin (Ig)G, mainly due to lower levels of IgG2, than non-smokers. The component(s) in tobacco smoke responsible for this effect is unknown, but animal studies have implicated nicotine as a major contributor to the immunologic effects of smoking. Does nicotine exposure due to use of smokeless tobacco (oral moist snuff) or nicotine replacement therapy influence serum Ig levels in humans?

Methods: Serum content of Ig classes and IgG subclasses was analysed in 77 non-smoking nicotine consumers, including 48 users of oral moist snuff (smokeless tobacco users) and 29 ex-smokers on nicotine replacement therapy, and compared with 44 healthy controls. Former smokers in any group had quit smoking at least 6 months prior to study entry. Ig class and IgG subclass levels were determined by radial immunodiffusion. Systemic nicotine exposure was excluded and confirmed by measuring urine content of cotinine using a quantitative radioimmunoassay.

Results: Ig class and IgG subclass levels did not differ significantly between the groups, with the sole exception of IgG4, which was significantly lower in nicotine consumers than in healthy subjects (0.4 ± 0.3 vs. 0.6 ± 0.4 g/l, mean \pm sd, 95% confidence interval $\{-0.3; -0.05\}$). There was no correlation between any Ig variable and cotinine concentration.

Conclusions: The decreased levels of IgG and IgG2 seen in tobacco smokers do not seem to be an effect of systemic exposure to nicotine.

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Introduction

Several large studies have shown that tobacco smokers have lower levels of immunoglobulin (Ig)G and IgA^{1–4} and higher levels of IgE^{5,6} than non-smokers, changes that may contribute to the propensity for respiratory infections seen in smokers. Among the IgG subclasses, IgG2 levels are affected most with a reduction of mean levels of

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approximately 40% compared with non-smokers.⁷ This is of special interest since smoking is an independent risk factor for invasive pneumococcal disease,⁸ and antibodies directed towards bacterial species commonly associated with respiratory infections (*Haemophilus influenzae* and *Streptococcus pneumoniae*) predominantly belong to the IgG2 subclass.^{9,10}

The mechanism(s) by which smoking affects serum levels of Ig has not been clarified. There is evidence of a dose-response relationship, with lower levels of IgG with increased smoking.^{2,4} Furthermore, the effect appears to be reversible after smoking cessation.^{2,11} The component(s) of cigarette smoke responsible for the effect has not been determined, but animal and in vitro studies have implicated nicotine as a major factor.^{12,13} However, whether serum levels of immunoglobulins are affected in non-smokers with systemic exposure to nicotine in a similar manner to that seen in smokers is not known. To date, no in vivo studies in humans relating to this issue have been presented. We therefore decided to perform a study of serum Ig and IgG subclass levels in non-smokers (ex-smokers or never-smokers) using oral moist snuff or nicotine replacement therapy (NRT).

Materials and methods

Design

This is a cross-sectional, parallel group study of serum Ig class and IgG subclass levels in nicotine consumers (NC), either users of oral moist snuff administered orally under the upper lip; smokeless tobacco users (STU), or ex-smokers with daily NRT. Healthy subjects with no exposure to nicotine served as a control group (HC). Participants were recruited by advertisements in two daily newspapers. Study size was based on the results of a previous study of the effect of smoking on serum levels of Ig.⁷

The study was performed at the Sahlgrenska University Hospital, Göteborg, and the study protocol was approved by the local ethics committee (Dnr S 26-01).

Study population

Subjects between 18 and 75 years of age were eligible. The study group consisted of current users of oral moist snuff, with or without a previous history of habitual tobacco smoking, and of ex-smokers who used nicotine on a daily basis by

means of a dermal plaster or chewing gum. Any former tobacco smokers had to have quit smoking more than 6 months before inclusion in the study.

The control group consisted of subjects who had never used nicotine at all or whose use had ended more than 6 months previously.

Subjects with diseases or medical treatments known or thought to influence serum Ig-levels were not included in the study. Accordingly, the following exclusion criteria were chosen: pregnancy; chronic liver or renal disease, diabetes mellitus, severe cardiac failure, severe chronic lung disease, any known immunodeficiency or rheumatologic disease; history of bronchial asthma, allergy or atopy; symptoms of infectious disease with pyrexia or any use of antibiotics, antihistamines or *N*-acetylcysteine during 4 weeks prior to the investigation. In addition, subjects with any use of steroids including oestrogen hormone substitution or any other immunomodulating treatment or vaccination during 2 months prior to the investigation were excluded.

Clinical examination and performance

Following an interview to ensure that all inclusion and no exclusion criteria were fulfilled, all subjects gave written detailed information of smoking history, use of snuff and NRT.

All subjects provided a 10 ml sample of venous blood, and a 10 ml urine sample. All samples were taken between 7 am and 10 am. Serum was separated, frozen and stored at -20°C awaiting further analysis. Urine samples were frozen and stored similarly. The study was performed between May and December 2001.

Determination of nicotine exposure

Systemic exposure to nicotine was assessed by a radioimmuno assay (RIA) with a double antibody for cotinine; a major metabolite of nicotine, the urine levels of which has been shown to accurately reflect systemic exposure to nicotine.^{14,15} A commercially available kit, Nicotine Metabolite Double Antibody DPC KTCD1, was used according to the instructions of the manufacturer (Diagnostic Products Corporation, Los Angeles, CA, USA). The qualitative cut off for a positive sample, i.e. systemic exposure to nicotine, with this RIA was $2.0\text{ }\mu\text{mol/l}$. The technical upper limit according to the standard-curve corresponded to a concentration of $85\text{ }\mu\text{mol/l}$. Higher values in a test sample were assigned this value. Subjects in the control

group had to have concentrations lower than 2.0 µmol/l.

Assay of serum immunoglobulins

The content of IgG, IgM and IgA as well as IgG subclasses in serum was assessed by radial immunodiffusion as previously described.⁷ For the definition of Ig class and IgG subclass deficiency, the class reference ranges of the department of Clinical Immunology (IgG 7.6–22.1 g/l, IgM 0.5–3.4 g/l and IgA 0.2–2.8 g/l) and the IgG subclass ranges published by Oxelius¹⁶ (IgG1 4.22–12.92 g/l, IgG2 1.17–7.47 g/l, IgG3 0.41–1.29 g/l and IgG4 <2.91 g/l) were used. The serum content of IgE was assessed using the ImmunoCAP™ method and the UniCAP™ kit of Pharmacia & Upjohn (Uppsala, Sweden) according to the instructions from the manufacturer. The result is given in units/ml with one unit corresponding to 2 ng. The upper limit for a normal concentration is 337 U/ml.

Statistical considerations

Most demographic and clinical data did not show a normal distribution and are presented as median values and ranges. For comparisons between groups of non-parametric data, the Mann–Whitney *U*-test or the χ^2 test with continuity correction according to Yates¹⁷ were used, as appropriate. Confidence intervals for the difference between median values were calculated according to Hollander and Wolfe.¹⁸ With the exception of IgE, immunoglobulin and cotinine data showed approximate normal distributions, and accordingly parametric methods were used. These data are presented as mean \pm SD. A Pearson or Spearman rank correlation coefficient was calculated to analyse correlations between quantitative variables. *P*-values <0.05 were accepted as significant.

Results

Subjects characteristics

A total of 125 recruited subjects were included and completed the study. Results from four subjects were subsequently excluded from final data analysis for the following reasons:

Two men in the control group had a complete IgA deficiency with a compensatory increase in IgG1 concentration. This condition is well recognized as the most common primary immunodeficiency in a caucasian population.¹⁹ One man in the control group had a high concentration of cotinine in urine (66 µmol/l). Finally, one man in the study group had no detectable cotinine in his urine sample.

Demographic and clinical data from the remaining 121 subjects, 77 NC, including 48 STU and 29 NRT, and 44 HC are presented in Tables 1 and 2. Gender as well as age distribution were similar in the two groups. Most controls (64%) were never-smokers while the opposite (83% ex-smokers) was true in the study group. Ex-smokers in the HC group had on average quit smoking longer ago and had smoked less than ex-smokers in the study group. In NC (Table 2) both the proportion of women and median age were higher in the NRT group.

Systemic nicotine exposure

STU had significantly higher levels of cotinine in urine than NRT. The correlation between consumption of nicotine containing products and cotinine levels in urine was investigated in the NRT and STU groups, respectively. The scattergram (not shown) revealed a reasonably strong correlation between daily dose of nicotine and cotinine in urine in the NRT group (Spearman; ρ 0.49, *P* = 0.009). In STU

Table 1 Demographic and clinical data in nicotine consumers and healthy controls.

	Nicotine consumers (<i>n</i> = 77)	Healthy controls (<i>n</i> = 44)	<i>P</i> -value	95% CI
Male/female	35/42	20/24	NS	—
Age (years)	44 (20–75)	43 (22–65)	NS	—
Number (%) of ex-smokers	64 (83)	16 (36)	<0.001	(30; 65)
Time since smoke-quitting* (years)	5 (0.5–20)	13 (1–25)	<0.001	(–13; –3)
Packyears*	20 (2–78)	8 (1.5–32)	<0.05	(2.3; 16.5)

Data are presented as median values with ranges in parenthesis. CI: confidence interval for the difference between groups. NS = not significant.

*Ex-smokers only.

Table 2 Clinical characteristics in nicotine consumers.

	Subjects on NRT (n = 29)	Smokeless tobacco users (n = 48)	P-value	95% CI
Male/female	9/20	26/22	<0.05	(-45;-1)
Age (years)	53(30-70)	39(20-75)	<0.001	(9;20)
Tobacco consumption (g/week)	—	88(12-525)	—	—
NRT dose (mg/day)	26(10-70)	—	—	—
Cotinine in urine (μmol/l)*	25 ± 16	37 ± 21	<0.05	(-21;-3)

Data are presented as median values with ranges in parenthesis with the exception of (*) where mean ± SD are shown. NRT: nicotine replacement therapy. CI: confidence interval for the difference between the groups.

Table 3 Serum levels of immunoglobulins (Ig) in nicotine consumers and healthy controls.

	Nicotine consumers (n = 77)	Healthy controls (n = 44)	Mean difference	95% CI
IgG (g/l)	10.5 ± 2.2	10.6 ± 2.0	-0.13	(-0.9;0.7)
IgA (g/l)	2.5 ± 1.1	2.3 ± 0.8	-0.21	(-0.2;0.6)
IgM (g/l)	1.4 ± 0.7	1.6 ± 0.6	-0.19	(-0.4;0.1)
IgG1 (g/l)	5.6 ± 1.6	5.9 ± 1.9	-0.26	(-0.9;0.4)
IgG2 (g/l)	3.4 ± 1.0	3.6 ± 1.1	-0.25	(-0.6;0.2)
IgG3 (g/l)	0.6 ± 0.3	0.6 ± 0.2	-0.02	(-0.1;0.1)
IgG4 (g/l)	0.4 ± 0.3	0.6 ± 0.4	-0.18	(-0.3;-0.05)
IgE (U/ml)*	17 (0-580)	25 (2-253)	-3**	(-10;4)

Immunoglobulin data are presented as mean ± SD with the exception of (*) where median values with ranges in parenthesis are shown. CI: confidence interval for the difference between the groups. (**) Median difference between groups.

the correlation was weaker (Spearman; ρ 0.36, $P = 0.015$).

Serum immunoglobulins and nicotine exposure

Immunoglobulin class and IgG subclass data are shown in Table 3. Mean differences in serum levels between the groups were small and with the exception of IgG4, which was significantly lower in the NC group, not statistically significant. Since the effect of smoking on Ig levels were found to be stronger in women than in men in a previous study⁷ women in each study group was compared separately. There were no significant differences for any Ig class or subclass between women in the NC and HC groups. The number of subjects with Ig or IgG subclass deficiency, as previously defined, is shown in Table 4. There were no statistically significant differences in proportions between the groups.

Within the NC group, subjects on NRT had significantly lower levels of IgG compared with the STU subgroup (9.5 ± 1.9 vs. 11.1 ± 2.2 g/l, mean difference -1.6 g/l, 95% CI $[-2.6; -0.6]$). There were no other significant differences between the two subgroups of nicotine consumers, either of Ig

Table 4 Number (%) of subjects with immunoglobulin deficiency according to Oxelius¹⁶ in nicotine consumers and healthy controls.

Immunoglobulin deficiency	Nicotine consumers (n = 77)	Healthy controls (n = 44)
IgG	4(5)	2(5)
IgG1	15(19)	4(9)
IgG2	0	0
IgG3	16(21)	8(18)
IgM	0	0
IgA	0	0

and IgG subclass levels, or of the fraction of subjects with Ig and IgG subclass deficiencies.

According to scattergrams (not shown) there was no apparent correlation between urine levels of cotinine and serum levels of any Ig variable in NC. All Pearson correlation coefficients were lower than 0.25.

Discussion

In this study we found only small and, with the exception of IgG4, insignificant differences be-

tween subjects with chronic nicotine exposure and subjects with no recent exposure. Although Ig levels did not differ much between the groups, there was a tendency towards lower levels in NC in all the measured Ig variables. While we cannot exclude that these small differences are the result of nicotine exposure, the relations between cotinine concentrations and levels of different Ig variables contradicts this possibility. This was found to be true also for IgG4, the only IgG subclass that was significantly lower in NC. These findings are in contrast with studies in smokers, where a clear dose–response relationship between daily cigarette consumption and IgG levels was found in two large studies.^{2,4} Whether the difference in IgG4 levels between the groups is a chance finding or an effect of nicotine exposure is unclear but the lack of a dose–response relationship favours the first interpretation. In short, nicotine does not seem to contribute to the effect on serum Ig levels that we see in smokers.

When analysing data, the issue of a possible gender difference was of particular interest to us. However, we found no indication of this when women and men in study and control groups were compared separately. Thus, the greater effect of smoking on serum levels of IgG and IgG2 in women than in men that we reported previously, was not found in non-smoking women consuming nicotine in this study.⁷

In previous *in vitro* studies, primarily in rodents, nicotine has been shown to have immunosuppressive effects. Both decreased proliferative response of T-lymphocytes and decreased induction of antibody-forming cells after stimulation with anti-CD3 have been described (reviewed by McAllister-Sistilli and co-workers¹³). However, in the only *in vivo* study in humans that we have found, use of nicotine-containing chewing gum in ex-smokers who recently quit smoking did not influence salivary levels of secretory IgA.²⁰ In the present study previous findings in laboratory animals was not confirmed. There are at least two possible explanations for this discrepancy. First, different doses of nicotine. In animal studies where nicotine exposure was found to significantly impair T-cell function, nicotine exposure was carefully adjusted to correspond to that of smokers of 20–40 cigarettes per day.^{21,22} However, in a study where nicotine was found to completely inhibit antibody response from rabbit spleen cells, nicotine levels were 1000-fold higher than those found in smokers.¹² Secondly, the duration of nicotine exposure is important. In humans the plasma half-time of nicotine is only approximately 30 min,²³ and although the main metabolite cotinine has a much

longer half-time, its biological activity on human cells is only 1% of that of nicotine.²⁴ *In vitro*, there is no conversion of nicotine to cotinine, and the experimental conditions in the referred studies are thus not comparable with *in vivo* exposure to nicotine in humans.

It is possible that the tendency towards lower serum Ig levels we see in NC is a lingering effect of smoking. We chose 6 months of smoking abstinence as a minimum requirement for participation in the study, which according to previous studies is ample time after smoke-quitting for changes in serum immunoglobulin levels induced by smoking to revert to almost normal compared with never-smokers.^{2,4,11} However, time since smoke-quitting was substantially longer in the 16 ex-smokers from the control group than in the 64 ex-smokers in the NC group and the proportion of ex-smokers in each group also differed (Table 1). Furthermore, comparison of the two subgroups of NC revealed that IgG was significantly lower in the NRT group with higher historic tobacco consumption (packyears, data not shown) and containing no never-smokers, than in the STU group. For these reasons, it is possible that the small difference we see between the study groups (NC and HC) is a lingering effect of previous tobacco smoking.

Another interesting point is that the overall results were similar both in subjects on NRT and in STU. Nicotine-containing chewing gums or dermal plasters contain no other chemical with potential immunological effect than nicotine. Oral moist snuff, on the other hand, is a smokeless tobacco product, which in addition to nicotine contains a large number of chemical substances, many of which exert toxic effects.^{25,26} Use of this product exposes the organism to many more toxic substances than nicotine. However, our results indicate that this form of tobacco use has no effect on serum Ig levels and consequently that exposure to inhaled tobacco smoke but not tobacco products per se is necessary for this particular immunologic effect to occur.

The fact that we did not include a control group of smokers might be considered a drawback with the study. However, the literature offers overwhelming proof of the association between tobacco smoking and decreased levels of IgG. Our aim was to study the possible effect of nicotine and with this aim we feel that the design, enabling a comparison between a group with verified chronic exposure to nicotine and a group in which recent nicotine exposure could be excluded, was appropriate. The crucial issue is whether the degree of systemic exposure to nicotine in our study group is comparable with that seen in tobacco smokers.

There are good reasons to believe that this is the case. First, several reports from other researchers have shown that nicotine and cotinine levels in both plasma and urine are comparable or higher in users of smokeless tobacco^{27–29}—and more specifically—users of Swedish oral moist snuff^{30,31} than in smokers. Secondly, urine cotinine levels in our study population of NC were on average higher than those reported in tobacco smokers in a study using the same radioimmunoassay that we did.³² It is therefore highly unlikely that low nicotine exposure is the reason why no effect on serum Ig levels was found in our NC group.

To conclude, in this study we found no relation between systemic exposure to nicotine and serum levels of immunoglobulins. This was true irrespective of whether nicotine was administered in the form of chewing gum, dermal plaster or in the form of smokeless tobacco products. Our results make it highly unlikely that the nicotine content of tobacco smoke is a major contributor to the reduction in serum IgG and IgG2 levels seen in smokers.

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References

- Gulsvik A, Fagerhoi MK. Smoking and immunoglobulin levels. *Lancet* 1979;1:449.
- Mili F, Flanders WD, Boring JR, Annett JL, Destefano F. The association of race, cigarette smoking, and smoking cessation to measures of the immune system in middle-aged men. *Clin Immunol Immunopathol* 1991;59:187–200.
- Wolfe WH, Miner JC, Michalek JE. Immunological parameters in current and former US Air Force personnel. *Vaccine* 1993;11:545–7.
- McMillan SA, Douglas JP, Archbold GP, McCrum EE, Evans AE. Effect of low to moderate levels of smoking and alcohol consumption on serum immunoglobulin concentrations. *J Clin Pathol* 1997;50:819–22.
- Burrows B, Halonen M, Berbee RA, Lebowitz MD. The relationship of serum immunoglobulin E to cigarette smoking. *Am Rev Respir Dis* 1981;124:523–5.
- Jensen EJ, Pedersen B, Schmidt E, Dahl R. Serum IgE in nonatopic smokers, nonsmokers, and recent exsmokers: relation to lung function, airway symptoms, and atopic predisposition. *J Allergy Clin Immunol* 1992;90:224–9.
- Qvarfordt I, Riise GC, Andersson BA, Larsson S. IgG subclasses in smokers with chronic bronchitis and recurrent exacerbations. *Thorax* 2001;56:445–9.
- Nuorti JP, Butler JC, Farley MM, et al. Cigarette smoking and invasive pneumococcal disease. Active Bacterial Core Surveillance Team. *N Engl J Med* 2000;342:681–9.
- Siber GR, Schur PH, Aisenberg AC, Weitzman SA, Schiffman G. Correlation between serum IgG-2 concentrations and the antibody response to bacterial polysaccharide antigens. *N Engl J Med* 1980;303:178–82.
- Freijd A, Hammarström L, Persson MAA, Smith CIE. Plasma antipneumococcal antibody activity of the IgG class and subclass in otitis prone children. *Clin Exp Immunol* 1984;56:233–8.
- Hersey P, Prendergast D, Edwards A. Effects of cigarette smoking on the immune system. Follow-up studies in normal subjects after cessation of smoking. *Med J Aust* 1983;2:425–9.
- Roszman TL, Rogers AS. The immunosuppressive potential of products derived from cigarette smoke. *Am Rev Respir Dis* 1973;108:1158–63.
- McAllister-Sistilli CG, Caggiula AR, Knopf S, Rose CA, Miller AL, Donny EC. The effects of nicotine on the immune system. *Psychoneuroendocrinology* 1998;23:175–87.
- Feyerabend C, Russell MA. A rapid gas-liquid chromatographic method for the determination of cotinine and nicotine in biological fluids. *J Pharm Pharmacol* 1990;42:450–2.
- Lawson GM, Hurt RD, Dale LC, et al. Application of urine nicotine and cotinine excretion rates to assessment of nicotine replacement in light, moderate, and heavy smokers undergoing transdermal therapy. *J Clin Pharmacol* 1998;38:510–6.
- Oxelius V-A. IgG subclass levels in infancy and childhood. *Acta Paediatr Scand* 1979;68:23–7.
- Altman DG. *Practical statistics for medical research*, 1st ed. London: Chapman&Hall; 1992. p. 235–42.
- Hollander M, Wolfe DA. Distribution-free confidence interval based on Wilcoxon's rank sum test. In: *Nonparametric statistical methods*. New York: Wiley; 1973. p. 78–82.
- Johansson SGO, Högman CF, Killander J. Quantitative immunoglobulin determination. Comparison of two methods. Estimation of normal levels and levels in persons lacking IgA and IgD. *Acta Pathol Microbiol Scand* 1968;74:519.
- Olson BL, McDonald Jr JL, Gleason MJ, et al. Comparisons of various salivary parameters in smokers before and after the use of a nicotine-containing chewing gum. *J Dent Res* 1985;64:826–30.
- Geng Y, Savage SM, Johnson LJ, Seagrave J, Sopori ML. Effects of nicotine on the immune response. I. Chronic exposure to nicotine impairs antigen receptor-mediated signal transduction in lymphocytes. *Toxicol Appl Pharmacol* 1995;135:268–78.
- Geng Y, Savage SM, Razani-Boroujerdi S, Sopori ML. Effects of nicotine on the immune response. II. Chronic nicotine treatment induces T cell anergy. *J Immunol* 1996;156:2384–90.
- Isaac PF, Rand MJ. Cigarette smoking and plasma levels of nicotine. *Nature* 1972;236:308–10.
- Fischer A, König W. Modulation of in vitro immunoglobulin synthesis of human peripheral blood mononuclear cells by nicotine and cotinine. *Clin Investigat* 1994;72:225–32.
- From the Centers for Disease Control and Prevention. Determination of nicotine, PH, and moisture content of six US commercial moist snuff products—Florida, January—February 1999. *J Am Med Assoc* 1999;281:2279–80.
- Idris AM, Ibrahim SO, Vasstrand EN, et al. The Swedish snus and the Sudanese toombak: are they different? *Oral Oncol* 1998;34:558–66.
- Russell MA, Jarvis MJ, Devitt G, Feyerabend C. Nicotine intake by snuff users. *Br Med J* 1981;283:814–7.
- Benowitz NL, Porchet H, Sheiner L, Jacob III P. Nicotine absorption and cardiovascular effects with smokeless

- tobacco use: comparison with cigarettes and nicotine gum. *Clin Pharmacol Ther* 1988;**44**:23–8.
29. Bolinder G, de Faire U. Ambulatory 24-h blood pressure monitoring in healthy, middle-aged smokeless tobacco users, smokers, and nontobacco users. *Am J Hypertens* 1998;**11**:1153–63.
 30. Holm H, Jarvis MJ, Russell MA, Feyerabend C. Nicotine intake and dependence in Swedish snuff takers. *Psychopharmacology* 1992;**108**:507–11.
 31. Andersson G, Bjornberg G, Curvall M. Oral mucosal changes and nicotine disposition in users of Swedish smokeless tobacco products: a comparative study. *J Oral Pathol Med* 1994;**23**:161–7.
 32. Hansen AM, Garde AH, Christensen JM, Eller N, Knudsen LE, Heinrich-Ramm R. Reference interval and subject variation in excretion of urinary metabolites of nicotine from non-smoking healthy subjects in Denmark. *Clin Chim Acta* 2001;**304**:125–32.