

# Mucosal immunodeficiency in smokers, and in patients with epithelial head and neck tumours

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## Abstract

Cigarette smoking influences the risk of orogastrointestinal disease in both protective (ulcerative colitis), and inductive (squamous tumours of the head, neck and oesophagus) roles. In order to study the effects of smoking on mucosal immunity, salivary immunoglobulins were measured in pure parotid saliva from groups of healthy non-smokers, smokers, and exsmokers and from patients with epithelial head and neck tumours, both untreated and after radiotherapy. Of the healthy individuals, smokers had significantly lower salivary IgA and higher IgM concentrations than did non-smokers. The effect on IgA was dose related, and reversible after cessation of smoking. Likewise, in patients with head and neck tumours (the majority being smokers), salivary IgA concentration was reduced and IgM increased when compared with non-smoking controls. Results were similar before and after radiotherapy. This study provides evidence of the effects of smoking on mucosal immunity as evaluated by parotid salivary immunoglobulins. Further studies of the influence of smoking on secretory immunity are indicated.

There is strong epidemiological evidence that smoking has a role in certain inflammatory and malignant orogastrointestinal diseases.<sup>1</sup> The incidence of ulcerative colitis is much lower in smokers than in non-smokers or exsmokers.<sup>2-4</sup> On the other hand smoking is strongly associated with epithelial tumours of the upper orogastrointestinal tract.<sup>1</sup> It is likely to be of relevance to these disease associations that cigarette smoking has profound effects on the immune system, and this has recently been reviewed.<sup>5</sup> Much work has been conducted on systemic immune status but, surprisingly, the influence of smoking on mucosal immunity has been relatively neglected. A recent report that smokers have reduced numbers of antigen presenting cells in the cervical epithelium<sup>6</sup> is of interest.

As part of a programme of research on human mucosal immunity, we developed techniques for the measurements of salivary immunoglobulin concentrations by ELISA. In order to investigate a possible effect of smoking on mucosal immunity we applied these techniques to stored saliva which had been collected in Edinburgh for other purposes from healthy smokers, healthy non-smokers, and patients with epithelial head and neck tumours. Results from these Scottish patients and healthy subjects revealed significant differences between the various groups. To confirm these data and in addition to avoid the possible confounding effects of concurrent

alcohol consumption, prospective studies were then performed in Egyptian, non-drinking, healthy subjects and patients with head and neck tumours.

## Methods

### EDINBURGH HEALTHY SUBJECTS AND PATIENTS

Healthy smokers and non-smokers were recruited from volunteers, and disease controls from patients attending clinics for obesity, bulimia, and treated hypo and hyperthyroidism. Patients with head and neck tumours were recruited from the Otolaryngology clinics. Patients who had received radiotherapy had been treated with a standard 52.5 Gy course over four weeks in 20 treatments. The field of irradiation did not include the parotid glands. Because of the retrospective nature of this phase of the study alcohol intake was not recorded, smoking history was incomplete for the non-smokers (never smoked or ex-smokers?) and the groups were not age and sex matched. Clinical details are given in Table I.

### CAIRO VOLUNTEERS AND PATIENTS

Healthy non-smokers, smokers, and ex-smokers were recruited from clinical, laboratory and ancillary staff, and from people attending for the removal of ear wax at the Ain Shames University Hospitals, Cairo, Egypt. In addition, all patients with epithelial head and neck tumours who were attending the Department of Otolaryngology in

TABLE I Subject numbers in each group from both centres. ? = status unknown (retrospective data - see text), -- = not investigated.

Subjects	Edinburgh			Cairo		
	M	F	Total	M	F	Total
Healthy controls						
Non-smokers	31	83	114	19	11	30
Smokers	19	9	28	27	20	47
Ex-smokers						
- 2 yr	-	-	-	11	10	21
Ex-smokers						
- 5 yr	-	-	-	10	7	17
Age + sex matched for Tumour patients	-	-	-	18	10	28
Tumour patients						
Untreated						
Current smokers	13	4	17	14	4	18
Ex-smokers	?	?	?	7	4	11
Non-smokers	?	?	?	0	1	1
Unknown	5	5	10	0	0	0
Total	18	9	27	21	9	30
Irradiated						
Current smokers	0	0	0	7	2	9
Ex-smokers	?	?	?	14	7	21
Non-smokers	?	?	?	0	0	0
Unknown	26	12	38	0	0	0
Total	26	12	38	21	9	30

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Accepted for publication  
4 July 1989

these hospitals were invited to participate. These patients received 50–60 Gy in 25–30 treatments over five to six weeks. The field of irradiation included the parotid glands. Healthy subjects and patients did not take alcohol, and non-smoking groups were separately age and sex matched for healthy smokers and for the tumour patients. Of the patients with tumours (untreated and irradiated respectively), 13 and 11 had tumour in the larynx, nine and eight in the hypopharynx, and eight and 11 in the oropharynx.

Of the Cairo volunteers and patients there were 47 smokers mean age 29.8 years, range 21–37 and 30 non-smoking controls, mean age 29.2 (21–36) (age comparison,  $U=665.5$ ,  $p=0.682$ ). Mean age of 30 tumour patients was 52.7 (31–64), and of 28 controls, mean 51.6 (34–61), with again no difference between the test groups and their controls in terms of age ( $U=366$ ,  $p=0.596$ ). The irradiated group were somewhat older than both the control and the tumour patients, mean age 59.0 (48–73) ( $U=187.5$ ,  $p=0.0004$  *v* controls;  $U=250.5$ ,  $p=0.0033$  *v* tumour patients). These patients had tumours in various sites which did not differ between groups ( $\chi^2=0.699$ , 2 df,  $p=0.71$ ).

There were no differences in the sex distribution between any group of Egyptian subjects, ( $\chi^2=1.82$ , 4 df,  $p=0.7712$ ).

#### CLINICAL PROCEDURES

Full informed consent was obtained in every case. For each subject details of age, sex, weight, smoking history, general health and, if appropriate, tumour site were recorded. Before collection of saliva the oral cavity was examined for evidence of local sepsis (gingivitis, pharyngitis, tonsillitis, dental abscess: if any of these conditions were present, the person was excluded from the study). Parotid saliva was then obtained using a simple modification of a Carlsson-Crittenden cup (J Harvey, Lochgilphead, Argyll, Scotland). The secretion of parotid saliva was stimulated by 5% citric acid given in four 0.5ml aliquots: collection of saliva was bilateral and simultaneous for two minutes (Cairo) or unilateral for five minutes (Edinburgh group). Sample volumes were measured by graduated pipette, and the samples stored at  $-70^\circ\text{C}$  until analysis. Flow rates were calculated from the volume in millilitres divided by the collection period in minutes, divided by the number (one or two) of parotid ducts from which saliva was collected.

#### ASSAY TECHNIQUES

Salivary concentrations of IgA, IgG, and IgM were assayed by a double sandwich ELISA technique. Briefly, microtitre plates M129A (Dynatech, Billingshurst, Sussex) were coated with one in 5000 goat antihuman IgA, IgG, and IgM. The plates were washed five times with 0.9% saline with 0.05% Tween 20 (Sigma, Poole, Dorset), blocked for 20 minutes with the washing solution with 1% adult bovine serum (Scottish antibody production unit, Carlisle, Lanarkshire), then pure parotid saliva was applied at a

1:2 dilution for IgG and IgM and a 1:5 dilution for IgA, 125  $\mu\text{l}$  being added to each well, and left for approximately eight hours at  $20^\circ\text{C}$ . After further washing five times, alkaline phosphatase conjugated goat antihuman IgA, IgG, and IgM (Northeast Biomedical Laboratories, Uxbridge, Middlesex) was added and incubated overnight at  $4^\circ\text{C}$ . Again after washing five times, paranitrophenyl phosphate at a concentration of 1 mg/ml in 10% diethanolamine buffer was added. As a standard control, World Health Organisation human serum immunoglobulin (1st international reference preparation, National Institute for Biological Standards, Pottery Bar, Herts) was used. The plates were read at optical density 405 nm on a Dynatech Microelisa Autoreader MR580. Total protein was measured using the Pierce method. Electrolytes were analysed on a standard SMAC II.

#### STATISTICAL ANALYSIS

The Mann-Whitney U test, Wilcoxon's paired rank-sum test, and Spearman's rank correlation coefficient were used for statistical analysis.

## Results

### SALIVARY IMMUNOGLOBULIN CONCENTRATIONS

#### Edinburgh

The results are shown in Table II. Salivary IgA concentrations were decreased in smokers, and in patients with head and neck tumours, both before and after irradiation. Conversely, IgM concentrations were increased in smokers, and in patients with head and neck tumours, with a few extremely high values in the postirradiation group. These results must be interpreted with caution as the age and sex distribution was distinctly different from the healthy controls.

#### Cairo

Healthy smokers had significantly lower salivary IgA concentrations (Fig 1), and higher salivary IgM (Fig 2), when compared with non-smokers. There was no influence of smoking on salivary IgG concentration, Figure 3. There was a strong inverse correlation between salivary IgA concentration and the number of cigarettes currently smoked daily (10–60), (Fig 4); however, no such relationship existed for salivary IgG, or IgM concentrations. Salivary flow rates were weakly but significantly correlated positively with the number of cigarettes smoked daily ( $r=0.291$ ,  $p=$

TABLE II Salivary immunoglobulin concentrations in the Edinburgh subjects. \* = significant difference from control values,  $p < 0.0005$  in all cases. I = insufficient saliva.

	Subjects			
	Non-smokers (n=114)	Smokers (n=28)	Tumour patients (n=27)	Irradiated patients (n=30)
IgA	145 (92)	44 (41)*	80 (61)*	87 (49)*
IgM	0.26 (0.4)	1.04 (0.5)*	0.077 (0.2)	3.2 (9.2)
IgG	3.7 (9.5)	0.07 (0.1)	I	I

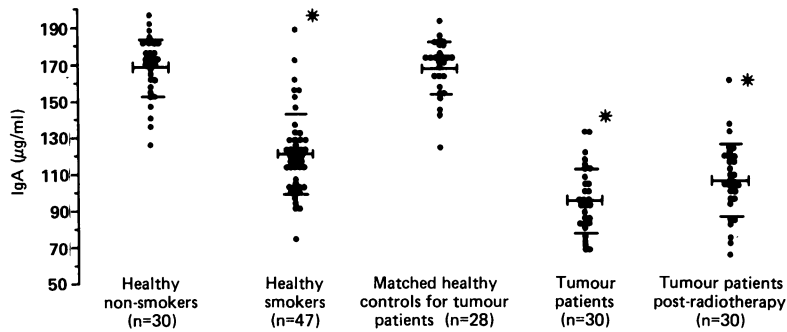


Figure 1: \* = significant difference between appropriate control groups and smokers and patients ( $U=84$ ,  $p<0.00001$  v healthy smokers;  $U=2$ ,  $p<0.00001$  v tumour patients;  $U=9$ ,  $p<0.00001$  v irradiated tumour patients). Bars show mean (standard error).

0.044) but no relationship existed between salivary immunoglobulin concentrations and flow rate.

Salivary IgA concentrations in exsmokers of at least 20 cigarettes daily, who had given up smoking two years before sampling, were higher than in healthy current smokers, and after five years of non-smoking IgA concentrations were similar to those of non-smokers (Fig 5). The effects of stopping smoking on salivary IgM concentrations were less consistent. Values were similar to those for non-smokers after two years' abstinence ( $U=267$ ,  $p=0.641$ , data not shown), but at five years were significantly higher than in non-smokers ( $U=91$ ,  $p=0.0004$ ).

There were identical findings when the patients with head and neck tumours (the majority being smokers, or very recent ex-smokers) were compared with the non-smoking controls (Figs 1-3). Again, salivary IgA concentration was markedly decreased and IgM concentration increased, IgG being normal, when compared with values for matched non-smoking controls. No differences were seen between the groups of patients who were untreated, and those who had undergone radiotherapy, Figures 1-3.

Identical results were found in both centres with no differences in salivary protein and electrolyte concentrations between any of the groups.

### Discussion

We have shown a striking and reproducible influence of cigarette smoking on salivary immunoglobulins. In healthy smokers, the concentration of IgA in pure parotid saliva is

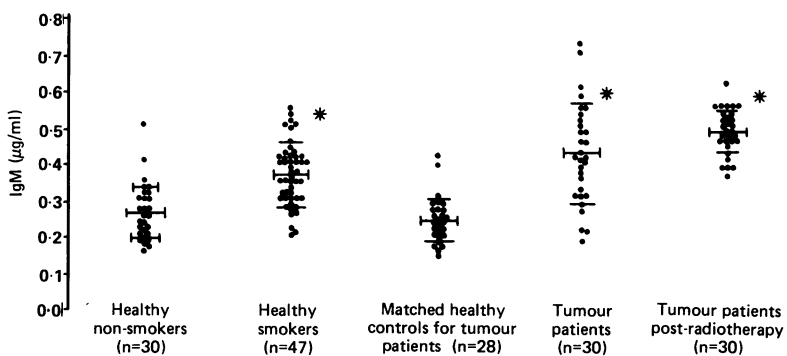


Figure 2: \* = significant difference between appropriate control groups and smokers and patients ( $U=250$ ,  $p<0.00001$  v healthy smokers;  $U=113$ ,  $p<0.00001$  v tumour patients;  $U=11$ ,  $p<0.00001$  v irradiated tumour patients). Bars show mean (standard error).

reduced, and of IgM increased, when compared with non-smokers. For IgA, this effect is strongly dose dependent, and reversible; salivary IgA concentrations were similar to those for non-smokers in a group of healthy subjects who had stopped smoking at least five years before. Differences in parotid salivary immunoglobulin concentrations between healthy non-smokers and patients with head and neck cancer might be attributed solely to the smoking habits of the latter. Such a relationship is blurred by the fact that many smokers are heavy alcohol drinkers,<sup>7</sup> and because alcohol consumption (in the absence of hepatic cirrhosis) increases salivary IgA concentrations (Barton and Ferguson, unpublished observations) and is an independent risk factor for squamous cell cancer of the head and neck.<sup>8</sup> We avoided this latter factor by using non-drinkers in the second, case controlled study in which identical results were obtained. Nevertheless in future studies it will be necessary to exclude other environmental factors such as drugs, and genetic factors which influence immunoglobulin levels.

The similarity of the findings between healthy smokers and patients with epithelial head and neck tumours probably reflects the fact that most people who develop these neoplasms smoke. Although many patients in the untreated tumour group and most patients in the postradiotherapy group had stopped smoking at the time of sampling, most commonly at the time of diagnosis, sufficient time may not have elapsed to allow any possible normalisation of salivary immunoglobulin levels.

We are aware of four previous reports of salivary immunoglobulins in smokers,<sup>9-12</sup> but they relate to small numbers of selected patients, and whole saliva was used. Even in the absence of oral sepsis or radiation damage, mixed saliva is contaminated with crevicular fluid, similar in its immunoglobulin content to serum, and is therefore unsuitable as material for the study of mucosal immunity.<sup>13</sup> This is highlighted by the discrepant results in the literature on the influence of smoking on immunoglobulin content of mixed saliva. Bennett found that salivary IgA concentration was low in eight elderly, edentulous male smokers,<sup>11</sup> in contrast with Watanabe who reported high mixed salivary IgA levels in a group of smokers and drinkers, and in head and neck cancer patients.<sup>9</sup> Hersey reported a striking decrease in salivary IgG (probably derived from crevicular fluid or inflammatory exudates) in a small group of healthy smokers who stopped smoking.<sup>10</sup> Olson found no change in whole salivary IgA when smokers stopped smoking and took nicotine or placebo chewing gum.<sup>12</sup> The safe and simple collection of pure parotid saliva provides a clean and stable secretory fluid for assay. This is particularly important in patients with head and neck tumours and after radiotherapy, when there may be immunosuppression and oropharyngeal superinfection.

Studies of serum and circulating leucocytes have been used to determine the effects of smoking on systemic immunity. Smokers have increased polymorphonuclear neutrophil counts, decreased natural killer cell activity, an increase in total T cell numbers with a decrease

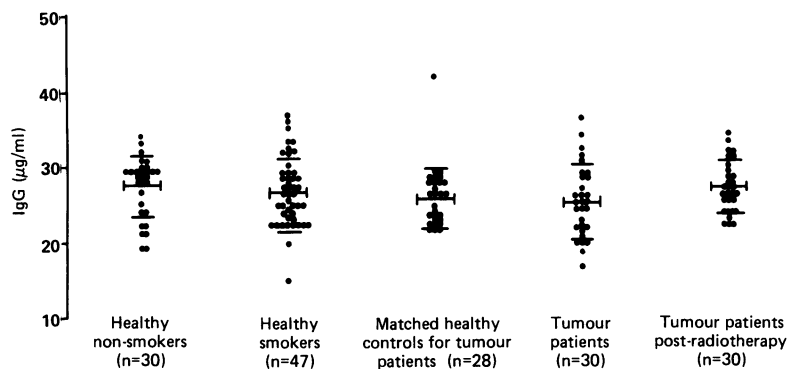


Figure 3: Bars show mean (standard error). No significant differences between groups, ( $U=566$ ,  $p=0.144$ , healthy smokers v controls).

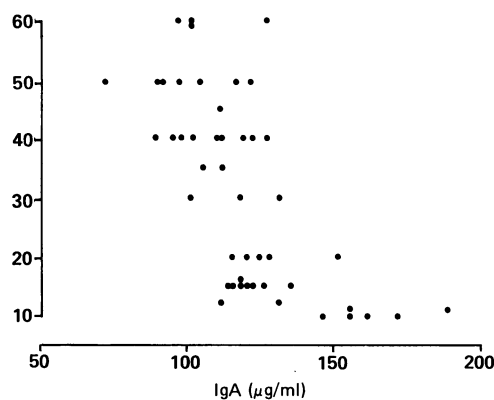


Figure 4: Relationship between daily cigarette consumption and salivary IgA concentration,  $r=-0.68$ ,  $p<0.00001$ .

in the T helper/suppressor cell ratio in heavy smokers. In smokers, serum IgA, IgG, and IgM are generally lower by 10–20% than in non-smokers, with higher concentrations of serum IgD and IgE in smokers.<sup>5</sup> Smokers have a generally decreased systemic response to antigens encountered at the nasal and respiratory mucosae,<sup>14,15</sup> although at the mucosal level conflicting results have been reported for total IgA in bronchoalveolar lavage fluid, with a decrease<sup>16</sup> and a three to four-fold increase<sup>17</sup> reported; at the nasal mucosa a decrease in local antibody levels in smokers is reported.<sup>18</sup> In a group of patients with head and neck tumours high whole salivary IgA concentrations were shown to correlate with tumour load, and decrease towards normal after successful treatment.<sup>19</sup>

The increase in IgM concentrations might be interpreted as a compensatory rise. This concept is a little naive; IgM has very different properties to IgA, and its higher concentrations may reflect immunopathology rather than simple compensation. We have not yet addressed the mechanism whereby cigarette smoking alters the immunoglobulin profile of saliva. Intuitively, we favour the concept that smoking impairs T cell immunoregulation of B cell differentiation and maturation, and propose to pursue this hypothesis in the first instance. Alternatively, the alterations may be mediated via the afferent limb of immunity, gut antigen presenting cells as recently reported for uterine cervical epithe-

lium.<sup>6</sup> Both this study and our own suggest a mechanism other than direct toxicity, as the cervix and parotid gland are distant from the local effects of cigarette smoke. It is probably unnecessary to confirm the self reported cigarette consumption; we have no reason to suppose that any errors in reporting would vary between light, moderate or heavy smokers, and others have shown self reported smoking habits to be reliable.<sup>20</sup>

Thus we have shown that smokers have a dose-dependant and probably reversible humoral mucosal immunodeficiency, as reflected either directly or otherwise by salivary IgA concentrations. Studies of mucosal immunity at other surfaces are necessary and should include investigation of T cell factors and other regulatory mechanisms. If changes in secretory immunoglobulins reflect abnormalities of other, as yet unexplored components of mucosal immunity, new avenues of pathogenesis of smoking related diseases may emerge.

We would like to thank Mrs Margaret Gordon and Mrs Jackie Johnston for excellent technical assistance. This work was supported in part by The Scottish Hospitals Endowments Research Trust (SHERT) and by the National Association for Crohn's and Colitis Disease (NACC).

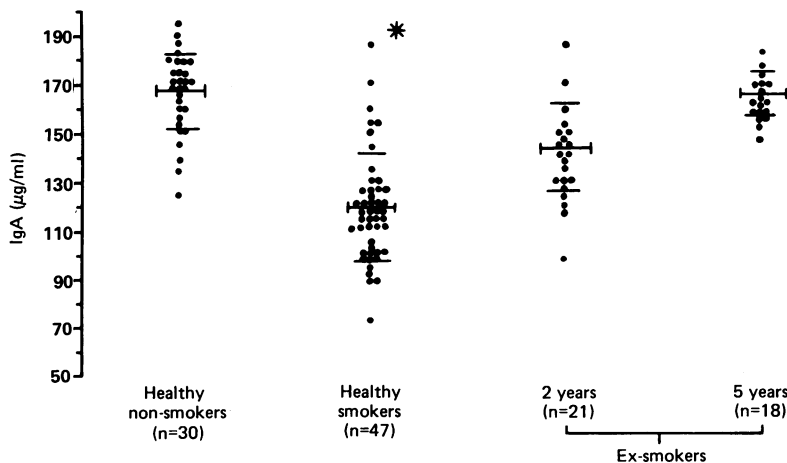


Figure 5: IgA concentrations in ex-smokers of 20 cigarettes daily, after 2 ( $U=102.5$ ,  $p=0.0001$ ), and 5 ( $U=214$ ,  $p=0.633$ ) years abstinence compared with controls. Current smokers are shown for comparison.

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