Inflammatory cell populations and cytokine mRNA expression in the nasal mucosa in aspirin-sensitive rhinitis

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ABSTRACT: Aspirin-sensitive rhinitis is characterized by severe perennial nasal congestion and discharge. The study questioned whether this disease, like immunoglobulin E-mediated rhinitis, might be associated with local recruitment and activation of T-lymphocytes, mast cells and eosinophils with parallel increases in "T-helper2-type" cytokines.

Nasal biopsies from 10 patients with aspirin-sensitive rhinitis and 12 healthy controls subjects were studied. Nasal mucosal sections were examined by immunohistochemistry in order to determine cell phenotypes and by *in situ* hybridization to detect cells expressing messenger ribonucleic acid (mRNA) for cytokines.

In aspirin-sensitive rhinitis there were increases in total (CD3+) ($p=0.05$) and activated (CD25+) T-cells ($p=0.007$), total (major basic protein (MBP) positive) ($p=0.004$) and activated (monoclonal antibody which recognizes the cleaved form of eosinophil cationic protein (EG2) positive) eosinophils ($p=0.003$), tryptase+ mast cells ($p=0.04$) and CD68+ macrophages ($p=0.002$). Neutrophils and cells expressing human leukocyte antigen-DR were no different. Marked increases were observed in the numbers of interleukin (IL)-5 mRNA+ cells ($p=0.004$) in aspirin-sensitive patients, whereas lower numbers of IL-4 mRNA+ cells were observed, with a trend for a difference from controls ($p=0.07$). No differences were observed for either IL-2 or interferon- γ .

In conclusion, in aspirin-sensitive rhinitis there is intense inflammation of the nasal mucosa characterised by T-lymphocytes, eosinophils and mast cells. The predominance of macrophages and disproportionate increase in interleukin-5 compared to interleukin-4 messenger ribonucleic acid expression suggests that factors other than "allergic" mechanisms may be important in this disease.

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Adverse reactions to aspirin have been reported almost as long as its clinical use as an analgesic and anti-inflammatory drug [1]. Symptoms of aspirin intolerance include rhinitis, urticaria, bronchial asthma, angio-oedema, purpura and anaphylaxis [2]. In patients with adverse reactions to aspirin, bronchial asthma and nasal polyps are frequently associated [3]. Intolerance to acetylsalicylic acid occurs in ~10–20% of adult asthmatics [4].

Although considerable progress has been made in understanding aspirin sensitivity as regards clinical diagnosis, biochemistry, cross-reactivity with nonsteroidal anti-inflammatory drugs (NSAIDs) and desensitization [5–7], the underlying mechanisms remain unclear. One proposal is that aspirin interferes with arachidonic acid metabolism by inhibiting the cyclooxygenase pathway and/or increasing the lipoxygenase-dependent release of leukotrienes (LTs) [8, 9]. However, this theory of "shunting" was questioned by the lack of an inverse correlation between lipoxygenase and cyclooxygenase (prostaglandin) products [6]. In bronchial biopsies from patients with aspirin-intolerant asthma, there is increased expression of the LTC₄ synthase gene [10]. This may be the result of a

mutation in the promoter region of the LTC₄ synthase gene [11]. Aspirin sensitive asthma is also associated with a marked increase in eosinophils in the bronchial mucosa [12, 13] and increases in LTs in nasal secretions [6] and in histamine and tryptase levels in serum [14, 15].

The role of a possible immunoglobulin (Ig)E-mediated mechanism in aspirin sensitivity remains controversial. For example, the time to onset of symptoms after oral aspirin intake (45–75 min) is more delayed than is typically observed for type I allergic responses [5]. Specific IgE antibodies to the metabolized products (but not the parent drug) of acetylsalicylic acid have been detected in the serum of patients with aspirin sensitivity [16, 17] by some, but not all investigators [4, 5].

Although several investigators have studied nasal polyps in aspirin-sensitive patients [18, 19], the nasal mucosa of patients with perennial rhinitis and aspirin intolerance has not been formally assessed. A previous study showed that patients with IgE-mediated seasonal allergic rhinitis demonstrate a typical immunopathology characterized by the presence of CD4+ T-lymphocytes, increased numbers of epithelial mast cells and eosinophils [20] and increases

in local expression of T-helper (Th)2-type (interleukin (IL)-4 and IL-5) but not Th1-type (IL-2 and interferon gamma (IFN- γ)) cytokines [21, 22]. In order to further explore the mechanism of aspirin-sensitive rhinitis, the cellular infiltrate and cytokine pattern in the nasal mucosa of patients with perennial rhinitis and aspirin intolerance compared to a matched group of normal healthy control subjects was assessed.

Materials and methods

Patients

Ten nonsmoking patients with aspirin sensitivity and symptoms of perennial rhinitis and 12 nonatopic normal healthy controls with no history of nasal or chest symptoms (other than common colds) were recruited from the Royal National Throat Nose and Ear Hospital and the Nose Clinic at Royal Brompton Hospital London, UK. The control subjects were the same as those employed in a previous study [23].

Inclusion criteria. Patients were recruited on the basis of: 1) perennial symptoms of sneezing, rhinorrhoea and nasal blockage for at least 2 yrs; and 2) a positive history of aspirin sensitivity with clear cut exacerbations of nasal symptoms with or without associated asthma symptoms within an hour of aspirin ingestion. Objective confirmation of aspirin sensitivity was obtained by intranasal lysine aspirin challenge in three patients for whom this test procedure was available. Oral aspirin challenges were not undertaken in view of the inherent risks of this procedure. All 10 patients had been diagnosed by a physician as having asthma and gave a typical history of asthma symptoms and response to inhaled bronchodilator. In general, their asthma was mild as confirmed by their normal median peak flow being 106% of predicted.

Exclusion criteria. Subjects were excluded if they gave a history of: 1) current pregnancy or breast feeding, 2) immunotherapy in the previous 5 yrs, 3) history of bleeding or clotting disorder, and 4) any other significant medical condition. Atopic patients were not excluded and 2/10 patients were noted to be atopic as defined by an immediate skin prick test response to one or more of a panel of aeroallergens (house dust mite, grasses, cat, dog, moulds) (Soluprick; ALK-Abello, Horsholm, Denmark). All other subjects were nonatopic according to these criteria. No patient had received aspirin or any other NSAID for at least 1 month prior to the nasal biopsy. All other medications including topical (intranasal or inhaled) glucocorticoids and sodium cromoglycate were discontinued for at least 2 weeks prior to biopsy. Antihistamines and inhaled bronchodilators were withheld for a minimum of 2 days and 12 h, respectively. The clinical data of the subjects participating in the study are shown in table 1.

Study design

The study was performed with the approval of the Royal Brompton Hospital Ethics Committee and the written informed consent of the subjects. For 2 weeks prior to

Table 1. – Clinical data of the subjects participating in the study

	Aspirin sensitive rhinitis patients	Normal control subjects
Subjects n	10	12
M/F	2/8	4/8
Age yrs	49 $\pm$ 15	32 $\pm$ 7
Asthma n	10	0
Nasal polyps n	10	0
PEF % pred	106	ND
Nasal symptom scores*	8.8	0
Asthma symptom scores ⁺	2.0	0
Atopy [#] n	2	0
Total IgE IU·mL ⁻¹	105 (36–146)	30 (17.5–95.0)

Data presented as mean $\pm$ SD or median (interquartile range) unless otherwise stated. *: Daily nasal symptoms (blockage, discharge, sneeze, itch) assessed on a severity score of 0–3, maximum score 12. ⁺: Daily asthma symptoms (cough, wheeze, breathlessness) assessed on a severity scale of 0–3, maximum score 9. [#]: $\geq$ 1 positive skin prick tests to inhaled allergens $>$ 3 mm in diameter. PEF: peak expiratory flow; IgE: immunoglobulin E; ND: not determined.

nasal biopsy, patients were asked to score (on a severity scale 0–3) daily symptoms of nasal blockage, discharge, sneeze and itch (maximal score=12). Patients were asked to record their daily asthma symptoms (cough, wheeze, breathlessness) on a severity scale (0–3) (maximal score=9) over the same time period. Patients then reattended for the nasal biopsy procedure. Local anaesthesia of the inferior nasal turbinate was achieved using 3% cocaine and 0.025% adrenaline. A 2.5 mm biopsy was taken precisely 10 min later using Gerritsma forceps as described [24]. Patients were observed for 1 h following the procedure and discharged with a contact telephone number.

Nasal biopsies

Biopsies were immediately cut in half and processed separately for subsequent *in situ* hybridization and immunohistology. Biopsy specimens were snap-frozen in isopentane pre-cooled in liquid nitrogen and stored at -80°C , pending analysis. Immunohistology was performed on 6 μm cryostat sections (fixed for 7 min in 60:40 acetone:methanol), using the modified alkaline phosphatase-anti-alkaline phosphatase method, as previously described [24].

Immunohistochemistry was performed using monoclonal antibodies (all from Dako Ltd., High Wycombe, UK) recognizing T-cells (CD45, CD3), activated T-cells (CD-25), T-cell subsets (CD4, CD3), human leukocyte antigen (HLA)-DR⁺ cells, macrophages (CD68), mast cells (AA1) and neutrophils (elastase). BMK13, the monoclonal antibody directed against major basic protein (MBP) was used to quantify total eosinophils, and was provided by J. Barkans (Dept of Allergy and Clinical Immunology, National Heart & Lung Institute, London, UK) and R. Moqbel (Pulmonology Research Group, University of Alberta, Canada). The EG2 monoclonal antibody (Kabia Pharmacia, Milton Keynes, UK), which recognizes the cleaved form of eosinophil cationic protein, was used to quantify the numbers of activated eosinophils.

Riboprobes, both antisense (complementary to messenger ribonucleic acid (mRNA) sequence) and sense (identical to the mRNA sequence), were prepared from

complementary deoxyribonucleic acid (cDNA) encoding IL-4, IL-5, IFN- γ and IL-2. cDNAs were inserted into different pGEM vectors (Promega, Southampton, UK) and linearized with restriction enzymes before transcription (IL-4: *Sph* I, *Eco* RI; IL-5: *Xba* I; IFN- γ : *Xba* I, *Eco* RI; IL-2: *Hind* III). Transcription was performed in the presence of 35 S-uridine triphosphate (S-UTP (Amersham, Buckinghamshire, UK); 12.5 μ L, 3.76×10^{13} Bq (1000 Ci)-mmol, for 1 h, at 37°C) and the appropriate T7 or SP6 (1 μ L, 20 units, for 1 h, at 37°C; Promega, Southampton, UK) ribonucleic acid (RNA) polymerase. *In situ* hybridization of 10 μ m cryostat sections was performed as previously described [25]. In order to minimize non-specific binding of 35 S, preparations were treated with 10 mM iodoacetamide and 10 mM *N*-ethylmaleimide for 30 min at 37°C and then in 0.5% acetic anhydride in (0.1 M) triethanolamine for 10 min at 37°C. Positive controls for IL-4 and IL-5 mRNA expression were cytopins prepared using a peripheral blood T-cell clone propagated from a patient with hyper-IgE syndrome. Positive controls for IFN- γ and IL-2 mRNA expression were cytopins prepared using phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells. For negative controls, nasal biopsy sections were: 1) hybridized with sense riboprobes for IL-4, IL-5, IFN- γ and IL-2, and 2) pre-treated with ribonuclease (RNase) A (20 μ g, for 1 h, at 37°C; Sigma, Poole, Dorset, UK) solution before hybridization with antisense riboprobes. Specific hybridization was recognized as clear dense deposits of silver grains in the photographic emulsion overlaying tissue sections.

Cell counts were performed with an Olympus BH2 microscope (Olympus Optical Company Limited, Japan) with a sidearm attachment linked to a computer graphics tablet (Summasketch Plus, Summagraphics Corporation, Fairfield, CT, USA) linked to a computer (Hewlett Packard International, Singapore). Cells in the submucosa were counted using an eyepiece graticule (microscope field area 0.202 mm²) aligned along the basement membrane to a

depth of 0.45 mm. Cell counts in the epithelium were expressed per square millimetre of epithelium employing the total cross-sectional area of the epithelium. The thickness of both the epithelium and the basement membrane was expressed in millimetres as the mean of five recordings at equidistant intervals along the whole length of the basement membrane.

Statistical analysis

Between-group comparisons were made using the Mann-Whitney U-Test. Correlations were performed using Spearman's rank method. For measurements of epithelial and basement membrane thickness, an unpaired Students t-test was used. All analyses were performed with the aid of a commercial software package (Minitab Inc., State College, PA, USA). A p-value <0.05 were considered significant.

Results

Immunohistology

Total cell counts were assessed using the pan-leukocyte marker CD45. There was no increase in cellularity in aspirin-sensitive rhinitis when compared to the submucosa of healthy controls ($p=0.3$). There were significantly higher numbers of both total (MBP+) and activated (EG2+) eosinophils (greater than 10-fold increases in median values, $p=0.0004$; $p=0.0003$). In contrast, neutrophil numbers were not significantly different ($p=0.3$) between the two groups. There was an approximate 2-fold elevation in T-lymphocytes (CD3+, $p=0.05$) and a marked increase in the numbers of CD25+ cells (presumed activated T-cells, $p=0.007$) in the aspirin-sensitive patients compared to normal control subjects (fig. 1). In contrast, median values for T-cell subsets (CD4+ and CD8+ cells) were not sig-

Fig. 1. — a) Total (BMK13) and b) activated (EG2+) eosinophils, c) T-lymphocytes (CD3+) and d) activated cells (CD25+) in the nasal submucosa (per microscope field 0.202 mm²) of aspirin-sensitive rhinitic patients and healthy control subjects. Results are expressed as number of cells per field at 200 $\times$ magnification. Median values are represented by solid bars. Levels of significance using the Mann-Whitney U-test are shown. Atopy had no impact on the differences in cell counts between aspirin-sensitive and normal control subjects, with the exception that if the two atopic patients (●) are excluded, the differences for CD3+ cells become insignificant ($p=0.15$). Circles with values in brackets represent data points which fall outside of the scope of the axes, the values are the number of cells per field.

Fig. 2. – Cytokine messenger ribonucleic acid (mRNA) expression for a) interleukin (IL)-5 and b) IL-4 in the nasal submucosa of aspirin-sensitive rhinitic patients and healthy controls. Results are expressed as number of mRNA positive cells per field (0.202 mm²) at 100× magnification. Median values are represented by solid bars. Between group comparisons were performed using the Mann-Whitney U-test ($p < 0.05$). If two atopic patients (●) were excluded, the differences for IL-5 were $p = 0.014$ and for IL-4 were $p = 0.13$.

nificantly different. Similarly, there was no increase in HLA-DR expressing cells (table 2). Aspirin-sensitive rhinitics also showed elevated numbers of mast cells ($p = 0.04$) and CD68+ macrophages ($p = 0.002$) compared to normal control subjects (table 2).

In situ hybridization

For patients with aspirin-sensitive rhinitis, there were considerably higher numbers of cells expressing IL-5 mRNA ($p = 0.004$) compared to control subjects. There was also a trend for higher numbers of IL-4 mRNA+ cells within the aspirin-sensitive group, although these values

Table 2. – Immunohistology of the nasal mucosa in aspirin sensitive rhinitics and healthy control subjects

Antibody	Aspirin sensitive rhinitis patient (n=9)	Normal controls (n=11)	p-value ⁺
CD45	118 (34.8–230)	65.8 (47.8–89.1)	0.31
CD3	49.6 (27.6–97.5)	21.5 (17.0–32.5)	0.05
CD4	44.5 (6.8–85.3)	22.2 (13.5–36.7)	0.65
CD8	36.9 (0.0–52.0)	3.8 (0.0–9.0)	0.30
HLA-DR	110 (0.0–144)	1.5 (0.3–2.5)	0.47
CD25	3.0 (1.1–13.6)	0.3 (0.0–1.2)	0.007
CD68 (macrophages)	42.3 (6.1–76.0)	1.8 (0.3–5.3)	0.002
MBP	13.0 (3.8–107)	0.2 (0.0–0.7)	0.0004
EG2	13.4 (4.6–104)	0.0 (0.0–0.9)	0.0003
Elastase (neutrophils)	10.0 (1.3–98.3)	3.8 (1.8–9.0)	0.30
Tryptase AA1	14.0 (10.4–18.4)	7.9 (2.2–11.7)	0.04

Data are presented as median cell counts (interquartile range) per field in the nasal submucosa for each antibody. ⁺: Between group comparisons using the Mann-Whitney U-test ($p < 0.05$). HLA-DR: human leukocyte antigen-DR.

Table 3. – *In situ* hybridization of the nasal mucosa showing number of cells expressing specific cytokine messenger ribonucleic acid

	Aspirin sensitive rhinitis patients (n=10)	Normal control subjects (n=12)	p-value ⁺
IL-5	12.5 (4.5–18.5)	0.0 (0.0–1.2)	0.004
IL-4	2.45 (0.0–4.7)	0.0 (0.0–1.8)	0.07
IL-2	2.15 (0.0–5.4)	0.5 (0.0–4.6)	0.70
IFN- γ	3.8 (0.15–6.0)	1.0 (0.0–2.3)	0.21

Data are presented as median cell counts (interquartile range) per field in the nasal submucosa for each cytokine. ⁺: Between group comparisons using the Mann-Whitney U-test ($p < 0.05$). IL: interleukin; IFN- γ : interferon gamma.

were lower than for IL-5 and did not achieve significance compared to normal control subjects ($p = 0.07$) (fig. 2). No differences were observed between aspirin-sensitive rhinitic and normal control subjects in the low numbers of cells expressing mRNA for the "Th1-type" cytokines IL-2 ($p = 0.7$) and IFN- γ ($p = 0.21$) (table 3).

Nasal epithelium

In aspirin-sensitive rhinitis, the nasal epithelium was significantly thicker than that observed for the normal control subjects (mean $\pm$ SEM 0.09 $\pm$ 0.01 versus 0.06 $\pm$ 0.005 mm, respectively; $p = 0.04$), although the integrity of the nasal epithelium was intact for both groups of subjects. This increase in thickness was also confirmed by an increase in the cross-sectional area of the epithelium (mm²) expressed per millimetre of basement membrane length for the aspirin-sensitive group (mean $\pm$ SEM 0.19 $\pm$ 0.03 mm²), compared to normal controls (0.10 $\pm$ 0.02 mm²) ($p = 0.01$). The thickness of the basement membrane (mm) did not differ between the two groups (mean $\pm$ SEM 0.014 $\pm$ 0.001 versus 0.015 $\pm$ 0.001 mm, respectively; $p = 0.4$). When cell counts in the nasal epithelium were assessed, there were significantly higher numbers of both mast cells ($p = 0.01$) and eosinophils ($p = 0.02$) seen for the aspirin-sensitive group (table 4).

Correlation between cell counts, cytokine messenger ribonucleic acid expression and nasal symptom scores

Daily nasal symptom scores were high in the aspirin-intolerant patients (median daily score 8.8, maximum

Table 4. – Immunohistology of the nasal epithelium in aspirin sensitive rhinitics and normal control subjects

Antibody	Aspirin sensitive rhinitis patients (n=10)	Normal control subjects (n=12)	p-value ⁺
MBP	2.2 (0–254)	0 (0–0)	0.02
EG2	4.0 (0–686)	0 (0–0)	0.02
Tryptase AA1	13.7 (0–62.4)	0 (0–0)	0.01
CD3	208 (124–325)	145 (78.4–237)	0.39

Data are presented as median cell counts (interquartile range) per square millimetre in the nasal epithelium for each antibody. ⁺: Between group comparisons using the Mann-Whitney U-test ($p < 0.05$).

range possible 0–12), whereas asthma symptoms were only modest (median daily score 2.0, maximum range possible 0–9). Normal control subjects scored 0 for both nasal and chest symptoms. For the aspirin-sensitive patients, the relationship between immunopathology and expression of clinical symptoms was assessed by correlating cell counts with the median daily diary symptom scores recorded over the 2 weeks immediately prior to nasal biopsy. A significant correlation was only observed between the degree of nasal blockage and the number of EG2+ activated eosinophils within the nasal epithelium ($r=0.67$; $p<0.05$).

Discussion

In aspirin intolerant patients with perennial rhinitis, high diary nasal symptom scores were associated with intense inflammation of the nasal mucosa even in the absence of recent exposure to aspirin. Compared to normal subjects the most significant differences were higher numbers of total and activated eosinophils, mast cells, activated T-cells and CD68+ macrophages and markedly higher numbers of cells expressing IL-5 mRNA. In contrast neutrophils, cells expressing HLA-DR and IL-4 mRNA+ cells, were not different from controls. These results are similar to those in the lower airways reported by PFISTER and coworkers [12, 13, 26] who found higher numbers of mast cells and eosinophils and increased expression of IL-5 in bronchial biopsies of aspirin-sensitive asthmatics. Similar increases in IL-5 but not IL-4 have been reported in bronchial biopsies taken during chronic isocyanate exposure in patients with occupational asthma [27].

These findings are in contrast to those recently reported during seasonal allergic rhinitis [21, 22]. Firstly, although increases in eosinophils, mast cells and IL-5 were observed during the grass pollen season, the number of cells in aspirin-sensitive asthma patients were 3–5 fold higher. Secondly, higher macrophage numbers were not observed during the pollen season in allergic rhinitis subjects. Thirdly, in seasonal rhinitis there were parallel increases in IL-4 and IL-5 mRNA+ cells, whereas in aspirin-induced rhinitis, few IL-4 mRNA+ cells were detected which failed to achieve significance when compared to normal control subjects. Fourthly, although the epithelium was intact in aspirin-sensitive rhinitis subjects, the thickness was increased in keeping with inflammation and oedema within the epithelium. Increases in mast cells and eosinophils and increases in IL-5 and IL-4 have also been reported in the nasal epithelium of patients with perennial allergic rhinitis [28]. Taken together these observations confirm a more intense IL-5 dominated mucosal inflammation in aspirin-sensitive rhinitis, distinct from that observed in IgE associated allergic rhinitis. In contrast to aspirin-sensitive rhinitis, the nasal mucosa in other non-allergic, noninfective cases of rhinitis without aspirin sensitivity (so-called "idiopathic" or "vasomotor" rhinitis), is characterized by the lack of an inflammatory component with no increases in mast cells or eosinophils [29–31].

In this study, the cell source of these cytokines was not colocalized and it remains to be determined whether the CD3+ T-lymphocyte is the major source as in allergic rhinitis, or whether mast cells and/or eosinophils are the

principal cells since both may produce IL-4 and IL-5 within the nasal mucosa [32, 33]. In contrast to HAMILLOS *et al.* [34] who reported significant increases in IFN- γ mRNA expressing cells within the nasal polyp tissue of aspirin-sensitive patients, the present results within biopsies of the nasal inferior turbinate could not be confirmed. Similarly, there were only occasional IL-2 mRNA+ cells and no difference between aspirin-sensitive patients and normal subjects.

In the treatment of aspirin induced bronchospasm, several authors have reported beneficial effects of lipoxigenase inhibitors [35]. LT release from eosinophils, mast cells and macrophages may contribute, at least in part, to the nasal obstruction observed in aspirin-sensitive rhinitis. In this regard, it is of interest that as in previous studies of patients with allergic rhinitis [24], a positive correlation between tissue eosinophil numbers and the symptom of nasal blockage was observed. Eosinophils are the dominant source of LTC₄, a potent inducer of airway narrowing in both the upper and lower airway [36]. Chronic inflammation of the nasal mucosa even in the absence of aspirin may be due to higher baseline levels of LTC₄ due to increased LTC₄ synthase as has recently been reported to be present in bronchial biopsies from aspirin-sensitive asthmatics [10].

In summary, this study has shown, in agreement with studies of the lower airways, that aspirin-sensitive rhinitis is an intense chronic inflammatory disease with infiltration of the nasal submucosa by eosinophils, mast cells, T-cells and increased IL-5 expression. The disparity between IL-4 and IL-5, and the findings of an intense macrophage infiltrate contrast with typical "allergic" nasal disease. The more prolonged (and severe) time course after aspirin ingestion and equivocal IgE data also question the role of "allergy" in this syndrome. Furthermore, cross-reactivity between NSAIDs with unrelated chemical structures would also argue against an immunological mechanism for aspirin-induced rhinitis. Similarly, cross-desensitization with other NSAIDs following successful aspirin desensitization would support a possible mechanism involving the cyclooxygenase pathway [15] or dysregulation of LTC₄ synthase which has been shown to be increased in the bronchial mucosa in aspirin-induced asthma [10].

A practical implication of these findings of intense eosinophilic inflammation, even in the absence of recent aspirin exposure, is the need for regular prophylactic treatment with anti-inflammatory agents. It is speculated that this might be achieved by topical corticosteroids as well as continued obsessional avoidance of aspirin and other non-steroidal anti-inflammatory drugs.

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