

Original article

Nasal lavage concentrations of free hemoglobin as a marker of microepistaxis during nasal provocation testing*

Background: The constituents of nasal mucus may be contaminated by plasma if there is epistaxis. Gross epistaxis is apparent as a red lavage fluid, while microepistaxis may yield a clear fluid. If gross or microepistaxis are present, it will be difficult to decide whether plasma protein concentrations are elevated because of plasma exudation or bleeding. In order to discriminate between these two possibilities, we measured erythrocyte-derived free hemoglobin (fHb) in nasal lavage fluids.

Methods: Single-blinded subjects underwent standard hypertonic saline nasal provocation. Unilateral hypertonic nasal provocation was performed in normal, allergic rhinitis (AR) and nonallergic rhinitis (NAR) subjects (total of 1316 specimens). fHb was measured using the Sigma-Aldrich kit (St. Louis, MO). Grossly bloody specimens were analyzed separately from the remainder. Statistical analysis defined the means and 95th percentiles for fHb and albumin in the nonbloody normal group.

Results: fHb concentrations ranged from below the limits of detection ($< 1 \mu\text{g/ml}$) to $> 164 \mu\text{g/ml}$ fHb was $79.3 \mu\text{g/ml} \pm 4.7$ (mean $\pm$ SEM) in four normal, 31 AR and 25 NAR grossly bloody specimens. The 95th percentile of fHb in the nonbloody normal samples ($n = 68$ subjects, $n = 681$ specimens) was $16.5 \mu\text{g/ml}$. This value was defined as the threshold to detect potential microepistaxis, and corresponded to approximately 245 000 erythrocytes per ml of lavage fluid. Total protein ($P < 0.05$) and albumin ($P < 0.001$), but not markers of glandular secretion, were significantly increased in samples with fHb $> 16.5 \mu\text{g/ml}$ compared to those $\leq 16.5 \mu\text{g/ml}$. Elevations of fHb without changes in albumin were more prevalent in nonallergic rhinitis.

Conclusions: Significant bleeding into nasal lavage samples can contaminate the specimens and increase the concentrations of both fHb and plasma proteins. Increased albumin alone would indicate increased vascular permeability. The mechanism(s) leading to elevated fHb without increased plasma proteins require further investigation.

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Nasal secretions contain proteins from the vascular system (a third of total protein), with two-thirds exocytosed from submucosal glands and epithelial cells (1). Nasal bleeding during provocation testing may contaminate lavage fluids and increase the concentrations of plasma proteins (albumin, fibrinogen, $\alpha 2$ -macroglobulin, immunoglobulin G, nonsecretory immunoglobulin A, and others). Also, in pathological states such as nasal polyposis (2), allergic rhinitis (AR) (3, 4), nonallergic rhinitis (NAR), and sinusitis (5),

epistaxis may be an essential component of the syndrome, or plasma proteins may be elevated because of mediator release and vascular leak without bleeding.

If bleeding occurs in the nose, free hemoglobin (fHb) will be released from erythrocytes that enter into the nasal cavity (6). We hypothesized that bleeding would increase the concentrations of fHb and albumin in epithelial lining fluid and nasal lavage fluid. If so, fHb in the nasal lavage samples could be used as a marker of microepistaxis and detect false elevations of plasma proteins in lavage samples. We set out to determine the range of fHb in normal subjects, and compared these levels to AR and NAR subjects. A threshold for normal fHb was defined as the 95th percentile for normal subjects. Grossly bloody specimens were apparent by

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inspection. Specimens that were not grossly bloody but that had elevated fHb were defined to have micro-epistaxis. As well, the relationships between fHb and albumin in each group were explored to determine if epistaxis was associated with AR or NAR.

Material and methods

Subjects and specimens

All participants gave informed consent to participate in this Institutional Review Board approved study. Nasal lavage samples were analyzed from 112 subjects who underwent unilateral hypertonic saline nasal provocation (7). These subjects were divided into 'normal' ($n=68$ skin test negative subjects) and 'rhinitis' ($n=44$ subjects) groups using 'positive Rhinitis Score' (RhSc) (see below). The 'rhinitis' group was further divided into AR ($n=38$ subjects) and NAR ($n=6$ subjects) based on positive allergy skin tests (at least two of 16 positive allergens) (8). The mean age (percentage of males) of normal, AR and NAR groups were 40 years (27%), 41 years (28%) and 43 years (18%), respectively. The numbers using nasal steroids and nonsteroidal anti-inflammatory drugs on a regular basis were determined for each group to determine if these drugs were risk factors for nasal hemorrhage.

In brief, pre-existing nasal secretions were removed by repeated normal saline (NS) lavage ($1 \times$ NS, 0.9% NaCl). Provocation with $1 \times$ NS (100 μ l) were then given into the right ipsilateral (IL) nostril followed by the left contralateral (CL) nostril. Five minutes later, 12 sprays of $1 \times$ NS were sprayed into the IL nostril. Lavage fluid was expelled by gentle blowing. The CL nostril was then lavaged separately. This cycle was immediately repeated with $3 \times$ NS (2.7% NaCl), $6 \times$ NS (5.4% NaCl), and $12 \times$ NS (10.8% NaCl), and then with $24 \times$ NS (21.6% NaCl) into the IL nostril. $1 \times$ NS (100 μ l) was always administered as a challenge into the CL nostril, and the CL side was lavaged separately each time. Specimens containing gel, sol and cellular components were gently homogenized then frozen for later analysis. A total of 1316 lavage samples were analyzed. Sixty were grossly bloody (red) while 1256 were clear.

Rhinitis questionnaire

A previously described Rhinitis Questionnaire (9) was used to assess symptoms of itchy nose, nasal fullness or congestion, sensation of blocked airflow, sneezing, discharge/runny nose, inability to breathe through nose, ear fullness, headache/pain in face, blowing out thick mucus, fever, postnasal drip in back of throat, and throat clearing/hoarse voice. Subjects scored the overall severity of these symptoms for the past six-month period on the four-point Likert scale as none (score=0), mild (score=1), moderate (score=2), or severe (score=3). The sum of the severity scores was the Rhinitis Score (RhSc). The maximum score was 36. Scores of 8 or higher were considered a 'positive RhSc' as previously defined (10).

Preliminary study to validate the relationship between erythrocyte number and fHb concentration

Approximately 10 ml of heparinized human peripheral blood was centrifuged at 2500 r.p.m., 4°C for 10 min, and the plasma and buffy coat removed. The erythrocytes fraction was diluted to 0.5% (1:50 000 dilution) and cell number counted on a Coulter Counter (Coulter Electronics Inc, Hialeah, Florida). The 0.5% erythrocyte solution contained 6×10^7 erythrocyte/ml. Dilutions of the erythrocyte solution or standard human fHb (5 μ l) were mixed with 25 μ l of pooled nasal lavage fluid known to have low fHb concentrations in 96-well microtiter plates. According to our usual protocol, plates were frozen overnight then thawed, a process that releases intracellular proteins from cells.

Measurement of free hemoglobin and other nasal constituents

We modified the standard Sigma–Aldrich method for measurement of nasal lavage fluid fHb in 96-well microtiter plates (NUNC, Copenhagen, Denmark). fHb was assayed in triplicate by mixing 25 μ l of nasal lavage fluids or standard human plasma fHb with 100 μ l of 3,3', 5,5'-tetramethyl-benzidine (TMB)–acetic acid solution (11). After adding 100 μ l of 0.3% hydrogen peroxide solution and incubating at room temperature for 5 min, absorbance was read at 630 nm. The assay range was linear from below 2.4 μ g/ml to 112 μ g/ml. Above 112 μ g/ml, the curve shifted to log-linear and eventually plateaued. Below 2.4 μ g/ml, the curve was linear to zero permitting extrapolation of the lowest values to 0 and 1 μ g/ml.

Nasal lavage specimens provoked by hypertonic saline were analyzed for total protein, albumin, lysozyme and mucin using previously published microtiter plate methods (12–14). Urea was measured by the Sigma kit with the following modifications for 96-well microtiter plates. After adding 175 μ l of blood urea nitrogen (BUN) reagent to 25 μ l of nasal lavage fluids or glucose/urea nitrogen combined standard and incubating at room temperature for 5 min, absorbance was read at 340 nm. The concentrations of nasal constituents were collated for specimens with fHb above and below 16.5 μ g/ml.

Histogram and statistical analysis

Histograms were constructed in EXCEL (Microsoft, Redmond, WA) to show the frequencies of each fHb concentration in the normal and rhinitis data sets. The limits of normal were found by eliminating values for grossly bloody samples. Values in the range of <1 –112 μ g/ml were transferred to STATISTICAL ANALYSIS SOFTWARE (SAS, Cary, NC) for statistical analysis. The distribution of fHb concentrations for the normal subjects (68 subjects with 681 nasal lavage specimens) was determined and the 95th percentile was calculated as the upper limit of normal. fHb values were then assessed by a general linear model (GLM) for multiple comparisons to determine if hypertonic saline provocation had any significant effect on fHb, and to compare normal and rhinitis data sets at each strength of hypertonic saline. Results for all normal, AR and NAR specimens were compared by ANOVA for multiple comparisons with Bonferroni correction for each variable. When significant differences were found, unpaired Student's *t*-tests were used to compare individual pairs of data. The same analysis was then performed for normal, AR and NAR specimens with $\text{fHb} \leq$ and >16.5 μ g/ml.

In order to assess the potential effects of bleeding, secretion concentrations of each analyte were measured for normal, AR and NAR specimens with $\text{fHb} > 16.5$ μ g/ml and compared by unpaired Student's *t*-tests to those ≤ 16.5 μ g/ml. This threshold was used for all normal and hypertonic saline specimens. Pearson's correlations (*r*) and expected variances (r^2) were determined for fHb and these

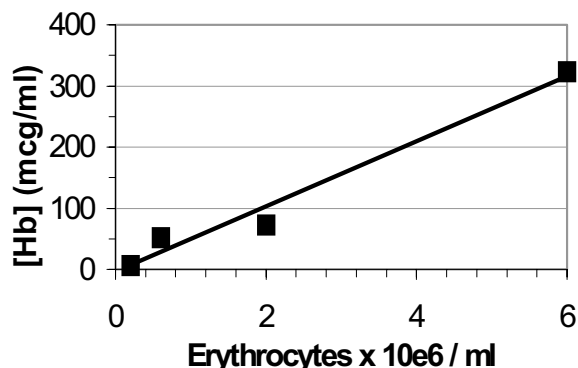


Figure 1. Graph showing the relationship between fHb and erythrocyte numbers per ml of nasal lavage fluid.

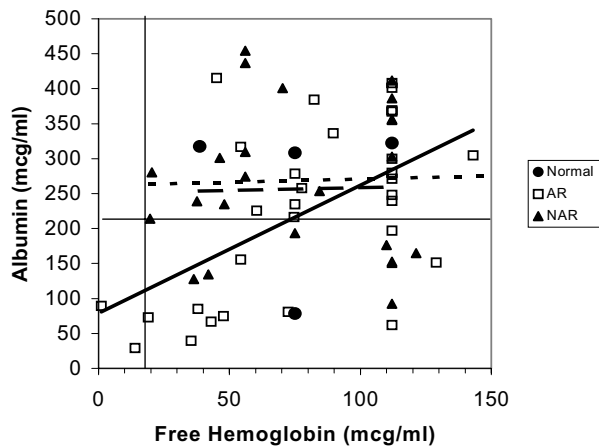


Figure 2. Scattergram showing the relationship between fHb and albumin in grossly bloody specimens. These analytes were correlated ($r^2=0.31$) for AR ($n=31$, open squares, solid line). Normal ($n=4$, solid circles) and NAR ($n=25$, solid triangles) showed no correlation for fHb and albumin ($r^2=0.0004$, dashed line, and $r^2=0.001$, dotted line, respectively). The solid vertical line represents the threshold for fHb. The solid horizontal line is the threshold for albumin. The graph showing the relationship between fHb and erythrocyte numbers per ml of nasal lavage fluid

other secreted macromolecules. In all cases, significance was ascribed for $P<0.05$. Data were reported as mean $\pm$ standard error of the mean (SEM).

The normal range of albumin concentration was also determined (95th percentile) so that elevations could be more clearly demonstrated graphically. The upper limits of normal for fHb and albumin were included on graphs of these variables. The numbers of specimens with normal or increased albumin and fHb were compared between rhinitis types by contingency tables.

Results

Rhinitis score

RhSc in the normal and rhinitis groups were 0.6 ± 0.2 , and 14.6 ± 1.2 , respectively ($P<0.0001$).

The relationship between erythrocyte number and fHb

The preliminary studies demonstrated that the freeze-thaw cycle released fHb from lavage fluid erythrocytes. The theoretical lowest limit of detection (X intercept) was 65 000 erythrocytes/ml (Fig. 1). Given the average erythrocyte density of 4.5×10^7 erythrocyte/ μ l of blood, this represents approximately 0.0014 μ l blood per ml of

nasal lavage fluid, a dilution factor of 6.9×10^9 . These data indicate the high sensitivity of the fHb assay for the detection of erythrocyte disruption in nasal lavage fluid.

Correlations of fHb and albumin in grossly bloody specimens

fHb and albumin were weakly but just significantly correlated in grossly bloody specimens from AR ($r^2=0.31$) but not normal ($r^2=0.0004$) or NAR ($r^2=0.001$) subjects (Fig. 2). These grossly bloody specimens were excluded from the analysis that followed.

Threshold value for an elevated fHb distribution

The 95th percentile for subjects with no rhinitis (RhSc <8) was 16.5 μ g/ml. This threshold value would be the result of 245 000 erythrocytes or 0.0054 μ l of blood per ml nasal lavage fluid. This threshold was used to segregate specimens without hemorrhage from those with potential microepistaxis or gross bleeding. Histogram analysis demonstrated that most samples in normal ($n=681$) and rhinitis ($n=575$) specimens (95%, and 88%, respectively) had very low fHb concentrations below the threshold value.

Serial hypertonic saline (IL side) provocation had no effect on fHb concentrations in either normal or rhinitis specimens ($P=0.9$) (Table 1). However, fHb was significantly higher in rhinitis compared to normal ($P=0.002$) (Table 1). Albumin concentration was not changed by the hypertonic saline in either group (data not shown). Previously we have shown that IgG, another plasma protein marker, was not altered. In contrast, lysozyme, mucin and urea were increased by $24 \times$ NS on the IL side only (7).

Comparisons of fHb for each rhinitis type

The concentration of fHb was 5.9 ± 0.4 μ g/ml when all specimens were compared (Table 2). Since results could vary due to the pathogenesis of the rhinitis type, the concentrations of analytes were determined for normal, AR and NAR groups. First, normal, AR and NAR results for all fHb, albumin, mucin and urea were found to be significantly separated by ANOVA for multiple comparisons with Bonferroni correction. fHb was highest in NAR. Albumin, mucin and urea were highest in AR.

Specimens with fHb ≤ 16.5 μ g/ml were similarly compared. Significant differences were found for fHb, albumin, and mucin. Unpaired Student's *t*-tests showed that fHb was significantly higher in NAR than normal ($P=0.003$) and AR ($P=0.02$). Albumin was significantly higher in AR than normal ($P=0.0001$) and NAR ($P=0.02$). Mucin was elevated in AR compared to normal specimens ($P=0.0001$).

In specimens with fHb >16.5 μ g/ml, albumin and urea showed significant differences by ANOVA for

Table 1. Effect of hypertonic saline provocation concentrations on fHb (mean $\pm$ SEM)

Group	1 x NS	3 x NS	6 x NS	12 x NS	24 x NS
Normal	3.7 ± 0.8	4.8 ± 1	5.1 ± 1.2	4.5 ± 0.9	4.6 ± 0.9
Rhinitis	8.7 ± 1.9	7.2 ± 1.2	5.8 ± 1.2	7.5 ± 1.5	8.8 ± 1.7

$P=0.9$ by GLM for comparison of all hypertonic saline strengths; $P=0.002$ for comparison of normal and rhinitis results.

Table 2. Mucus components ($\mu\text{g/ml}$) in normal, AR, and NAR specimens (mean $\pm$ SEM)

	Group	n	fHb	Albumin	Total protein	Lysozyme	Mucin	Urea
All specimens	All subjects	1256	5.9 $\pm$ 0.4	94.3 $\pm$ 3.9	627 $\pm$ 52.3	43.6 $\pm$ 2.1	462 $\pm$ 16.3	14.3 $\pm$ 0.2
	Normal	681	4.6 $\pm$ 0.4 ^{a,b}	76.1 $\pm$ 3.5 ^c	539 $\pm$ 27.5	39.8 $\pm$ 1.4	403 $\pm$ 19.8 ^c	13.6 $\pm$ 0.3 ^b
	AR	492	7.3 $\pm$ 0.7 ^a	123 $\pm$ 8.6 ^{a,c}	792 $\pm$ 127	50.6 $\pm$ 4.9	548 $\pm$ 29.3 ^c	15.1 $\pm$ 0.3 ^b
	NAR	83	9.5 $\pm$ 1.8 ^b	67.6 $\pm$ 7.8 ^a	372 $\pm$ 35.1	32.5 $\pm$ 3.1	422 $\pm$ 55.3	14.6 $\pm$ 0.6
	Normal	647	2.4 $\pm$ 0.1 ^e	72.3 $\pm$ 3.4 ^f	516 $\pm$ 26.2	39.7 $\pm$ 1.4	401 $\pm$ 20.4 ^f	13.7 $\pm$ 0.3
	AR	435	2.5 $\pm$ 0.2 ^d	101 $\pm$ 7.6 ^{d,f}	696 $\pm$ 136	50.5 $\pm$ 5.6	532 $\pm$ 29.2 ^f	14.8 $\pm$ 0.4
	NAR	69	3.5 $\pm$ 0.5 ^{d,e}	55.6 $\pm$ 7.5 ^d	332 $\pm$ 34.7	33.4 $\pm$ 3.7	426 $\pm$ 66.0	14.4 $\pm$ 0.7
	Normal	34	45.6 $\pm$ 4.8 ⁱ	136 $\pm$ 20.2 ^{h,i}	973 $\pm$ 222 ⁱ	40.7 $\pm$ 5.4	439 $\pm$ 81.2	12.2 $\pm$ 0.9 ^{g,i}
	AR	57	43.5 $\pm$ 3.5 ⁱ	295 $\pm$ 40.2 ^{g,h,i}	1518 $\pm$ 348 ^j	51.5 $\pm$ 5.0	669 $\pm$ 118	17.7 $\pm$ 0.9 ^{k,i}
	NAR	14	39.3 $\pm$ 5.8 ⁱ	127 $\pm$ 21.3 ^{g,i}	589 $\pm$ 103 ^k	27.9 $\pm$ 5.1	398 $\pm$ 43.1	15.8 $\pm$ 1.1 ^g

^a $P < 0.001$, ^b $P < 0.0005$, ^c $P < 0.00001$ by unpaired Student's *t*-tests for each variable in specimens between denoted normal, AR and NAR groups.

^d $P < 0.05$, ^e $P < 0.01$, ^f $P < 0.001$ by unpaired Student's *t*-tests for each variable in specimens between denoted normal, AR and NAR groups with fHb $\leq 16.5 \mu\text{g/ml}$.

^g $P < 0.05$, ^h $P < 0.01$, ⁱ $P < 0.001$ by unpaired Student's *t*-tests for each variable in specimens between denoted normal, AR and NAR groups with fHb $> 16.5 \mu\text{g/ml}$.

^j $P < 0.05$, ^k $P < 0.01$, ^l $P < 0.001$ by unpaired Student's *t*-tests for comparisons of each N, AR and NAR result between the fHb ≤ 16.5 and $> 16.5 \mu\text{g/ml}$ groups.

multiple comparisons with Bonferroni correction. Unpaired Student's *t*-tests showed that albumin was significantly higher in AR than normal ($P = 0.004$) and NAR ($P = 0.04$). Urea was elevated in AR ($P = 0.0003$) and NAR ($P = 0.04$) compared to normal specimens.

In order to assess the potential effect of bleeding for each rhinitis type, analyte concentrations were compared for fHb $> 16.5 \mu\text{g/ml}$ to those with fHb $\leq 16.5 \mu\text{g/ml}$ by unpaired Student's *t*-tests. In normal subjects, total protein ($P = 0.0003$) and albumin ($P = 0.0001$) were significantly higher in blood contaminated (fHb $> 16.5 \mu\text{g/ml}$) than clear samples. Similar results were found in NAR for total protein ($P = 0.007$) and albumin ($P = 0.00035$). In AR, total protein ($P = 0.038$), albumin ($P < 0.0001$) and urea ($P = 0.007$) were significantly higher in the microepistaxis (fHb $> 16.5 \mu\text{g/ml}$) group. In contrast, the glandular proteins, lysozyme and mucin, were not different,

indicating bleeding was not a significant source for lysozyme or mucin in nasal lavage fluids. Total protein, lysozyme, mucin and urea results were shown for comparison purposes but must be interpreted with caution since the levels secreted after $24 \times \text{NS}$ on the IL side were significantly higher than $1 \times \text{NS}$. The $24 \times \text{NS}$ specimens represented less than 10% of all specimens. Despite this potential bias, there were no changes in glandular markers. Albumin concentration did not change with hypertonic provocation (7).

Correlations of fHb and other mucus constituents

It was anticipated that there would be a close correlation between fHb and albumin. Therefore Pearson's correlations were calculated between fHb and each of the other variables. When all 1256 nonbloody specimens were used, the highest correlation was between fHb and albumin ($r = 0.33$). This indicated that only 11.2% (r^2) of the expected variances between fHb and albumin could be explained by the

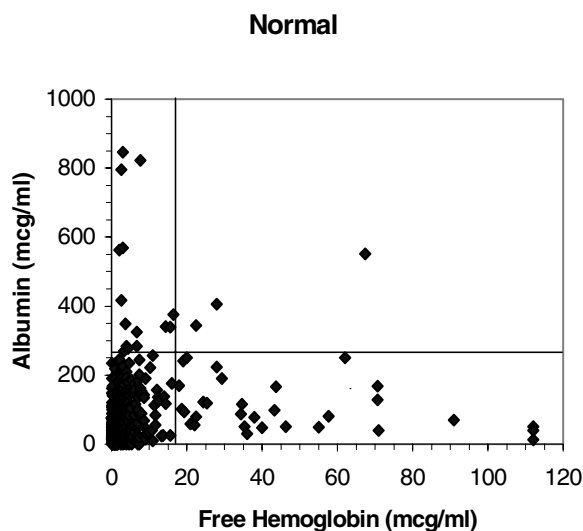


Figure 3. Scattergram showing the relationship between fHb and albumin in normal nonbloody specimens. Albumin and fHb were not correlated ($r^2 = 0.03$).

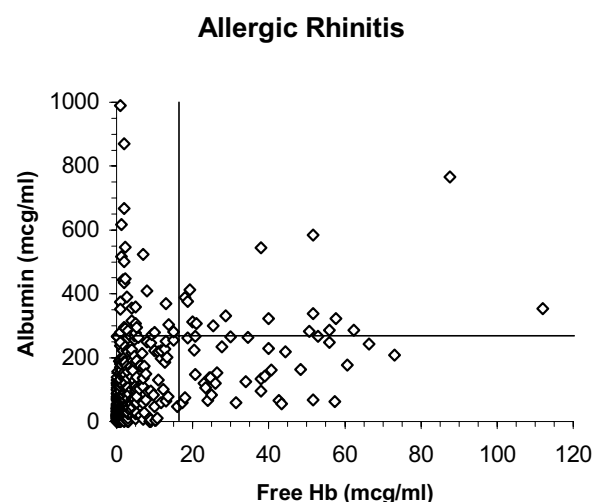


Figure 4. Scattergram showing the relationship between fHb and albumin in nonbloody allergic rhinitis specimens. Albumin and fHb were very poorly correlated ($r^2 = 0.18$).

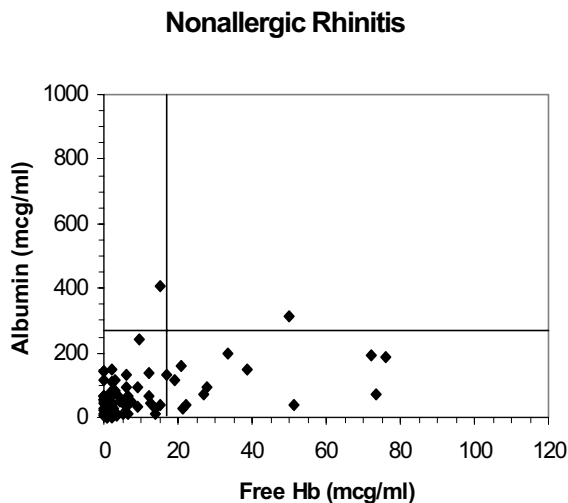


Figure 5. Scattergram showing the relationship between fHb and albumin in nonbloody nonallergic rhinitis specimens. Albumin and fHb were very weakly correlated ($r^2=0.20$).

interaction of these variables. In specimens with $\text{fHb} \leq 16.5 \mu\text{g/ml}$, the fHb and albumin correlation was 0.05 (r^2). When fHb was $> 16.5 \mu\text{g/ml}$, expected variances with total protein ($r^2=0.09$), albumin ($r^2=0.07$) and mucin ($r^2=0.06$) were again in the same low range, and indicated that there were no correlations between these independent variables in these samples.

The surprisingly low expected variances between fHb and albumin were further assessed for normal, AR and NAR groups. In the normal group, the 95th percentiles were calculated for fHb ($16.5 \mu\text{g/ml}$) and albumin ($260 \mu\text{g/ml}$). As previously mentioned, hypertonic saline provocation does not change albumin concentrations using our methods (7). These two thresholds divided graphs of fHb vs albumin concentrations into four quadrants. In normal specimens 93% (632/681) had normal albumin and fHb values (Fig. 3). Increased fHb with normal albumin was found in 5% (31/681). Increased albumin with normal fHb was present in 2% (15/681), while only 0.4% (3/681) were positive for both indices. The expected variance for all normal samples ($r^2=0.03$) indicated no correlation.

In AR, 81% (397/492) were in the lower left 'double normal' quadrant (Fig. 4). Increased fHb with normal albumin was found in 6% (31/492). Increased albumin with normal fHb was present in 8% (38/492). 'Double positive' specimens (upper right quadrant) accounted for 5% (26/492) of AR samples. AR specimens showed a modestly stronger correlation ($r^2=0.18$) for albumin and fHb.

Albumin and fHb were also modestly correlated ($r^2=0.20$) for NAR. Increased fHb was present in 17% (14/83, two right quadrants) while albumin was increased in only 2.4% (upper two quadrants) (Fig. 5).

The distributions of specimens between the four

quadrants shown on Figs 3–5 were significantly different ($P < 0.000001$, multiple contingency tables). There were differences between normal and AR ($P < 0.000001$), N and NAR ($P = 0.004$), and AR and NAR ($P = 0.0009$).

Drug treatment effects

Nasal steroids were used by 26% of AR, 17% of NAR, and 6% of normal subjects. fHb was higher in AR subjects who used nasal steroids ($19.6 \pm 7.7 \mu\text{g/ml}$) compared to those who did not ($7.8 \pm 2.6 \mu\text{g/ml}$) but the difference was not significant ($P = 0.067$, ANOVA). Steroid-treated NAR ($1.3 \pm 0.8 \mu\text{g/ml}$) and other NAR ($15.4 \pm 4.8 \mu\text{g/ml}$) fHb levels were not statistically different. Similarly, in normal subjects, steroid treatment was associated with a lower, but not significantly different, fHb ($1.9 \pm 1.4 \mu\text{g/ml}$ vs. $8.5 \pm 2.1 \mu\text{g/ml}$). When all subjects' fHb levels were pooled, there was no difference between those on nasal steroids and the remainder. The nonsignificant trends were continued when the percentages having $\text{fHb} > 16.5 \mu\text{g/ml}$ were compared.

Since nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used and can contribute to hemorrhage, the distribution of their use and relationship to fHb was assessed. NSAIDs were used by 21% of AR, 30% of NAR, and 10% of normal subjects. Mean fHb levels ranged from $7.6 \pm 5.3 \mu\text{g/ml}$ to $14.6 \pm 5.2 \mu\text{g/ml}$ in the NAR and AR groups, and there were no differences due to NSAID use. However, in normal subjects, NSAID use increased fHb levels ($25.7 \pm 15.2 \mu\text{g/ml}$ vs. $6.4 \pm 1.5 \mu\text{g/ml}$, respectively, $P = 0.005$). Use of the threshold value to discriminate between no hemorrhage and fine gross plus microepistaxis did not yield statistically significant values. For both drug treatments, statistical differences were minimal because of the wide ranges of fHb levels that resulted from the inclusion of gross epistaxis samples in the analysis. Only 2.7% of subjects used both nasal steroids and NSAIDs.

Discussion

fHb can be rapidly assayed in nasal lavage fluid by this simple method. The threshold for fHb associated with nonbloody samples using our methods was $16.5 \mu\text{g/ml}$. Specimens with values greater than this threshold were assumed to be potentially contaminated by hemorrhage. Comparison of bloody and nonbloody samples showed significant elevations of total protein and the plasma protein, albumin, in bloody samples. This indicated that a microhemorrhage could falsely elevate the apparent concentrations of lavage constituents such as fibrinogen, $\alpha 2$ -macroglobulin, and others (3, 4), and verifies the utility of screening with fHb measurements.

The threshold concentration of 16.5 µg/ml applies only to our lavage technique, and will vary depending upon the volume of lavage fluid and methods employed.

The statistical analysis was based on individual lavage specimens, and not the entire set of lavage specimens collected from any given individual during a provocation. Only the 10% of the specimens derived from 24 × NS provocations on the IL side were expected to show increased lysozyme, mucin or urea (7). This secretory effect did not alter the statistical outcomes. Therefore, the consequences of increased fHb levels could be clearly discriminated from any effects due to the specimen inclusion.

fHb was elevated by nasal steroid treatment in AR subjects and by NSAIDs in normal, nonrhinitis subjects. These results should be interpreted with caution. Verification of these trends will require recruitment of much larger numbers of subjects per disease group to reach sufficient statistical power.

The increased concentrations of albumin in AR specimens with fHb ≤ 16.5 µg/ml likely reflect underlying pathological conditions due to histamine-induced vascular permeability (Fig. 4)(15, 16). The significant increase of mucin in AR has been previously noted (15) and could be the result of induction of mucin gene expression by IL-9 (17), IL-13 (18), EGF (19) or mucous cell exocytosis by leukotrienes (20) or augmented parasympathetic reflex activity (21).

NAR subjects had higher fHb levels than normal and AR subjects in the subset with fHb ≤ 16.5 µg/ml. This suggests that mucosal friability, irritation, or other changes could lead to a higher level of microepistaxis that may be related to the mechanism(s) of NAR pathogenesis.

The correlation between fHb and albumin was weak for normal subjects. However, this was expected since the percentages of specimens above the normal thresholds (95th percentiles) were < 5% (lower left quadrants of Figs 3–5). There was overlap of clear samples with fHb > 16.5 µg/ml and grossly bloody samples with low fHb levels (Fig. 2). The fHb test is based on a peroxidase reaction. Falsely low fHb test results could occur despite the presence of active epistaxis if nasal secretions contained significant concentrations of endogenous antioxidants such as uric acid (22). For example, ascorbic acid inhibits the action of peroxidase and gives false negative test results (23).

Correlations were higher in AR and NAR since they had large percentages of outliers showing increased fHb and normal albumin (lower right quadrants of Figs 4 and 5). The increase in fHb could be the result of past epistaxis with clot formation and adhesion followed by slow clot disintegration with the release of fHb in the absence of any mechanism that could increase vascular permeability or albumin exudation. Bleeding into the interstitial space without tearing the epithelium is also possible. Erythrocyte lysis could lead to a gradual loss

of fHb across the epithelium as a part of the normal transepithelial flux of interstitial macromolecules. Several samples gave elevated fHb but were not grossly bloody. This potentially false positive result could occur in specimens with elevated neutrophil or eosinophil peroxidase concentrations since the peroxidase could potentially degrade the TMB substrate and generate the coloured reaction product. This is highly unlikely since there is approximately 10 pg myeloperoxidase (MPO) per neutrophil in airways (24, 25). In data not shown, we found that 16 000 ng/ml MPO would be required to falsely react with the fHb reagents to give an apparent fHb concentration of 16.5 µg/ml. This corresponds to approximately 1.6×10^6 neutrophil/ml lavage fluid. Lim et al. (26) found approximately 0.12×10^6 neutrophil/ml in baseline lavage fluid in active AR rising to 0.4×10^6 after allergen provocation. This would yield a fHb concentration of < 4 µg/ml. It is unlikely that MPO or neutrophil can cause false positive fHb readings. Specimens with increased albumin but normal fHb (upper left quadrant) could represent subjects studied during the immediate phase of allergic reactions (16), the first three days of rhinovirus infections (27), errors in assay technique or subject diagnosis (e.g. allergic subjects in the normal group). Elevation of both albumin and fHb (upper right quadrant) could be the result of active bleeding or clot lysis combined with vascular permeability due to mediator release (e.g. histamine, bradykinin) and may be related to the mechanism(s) of rhinitis (16).

The study was designed to establish a rapid method to detect the possibility of bleeding by the release of fHb from any of these potential sources. Because of the possibility that erythrocytes could be lysed in the lavage fluid, we did not attempt to correlate fHb and lavage fluid cell pellet erythrocyte numbers. If blood was lost, then albumin and erythrocyte or fHb should have been more closely correlated. As well, all specimens were frozen then thawed, which would lyse erythrocytes releasing their contents. A correlation would be expected for active bleeding, but could be absent with the other conditions listed above. This conclusion was supported by the skewed fHb results found with normal albumin levels (right lower quadrants of Figs 3–5) which showed increased fHb without an increase in plasma albumin exudation.

We conclude that the threshold value of 16.5 µg/ml for fHb using our lavage technique will exclude samples with hemorrhage. This may improve interpretation of nasal provocation tests by reducing the likelihood of inadvertent plasma contamination, and by identifying microhemorrhage as a potential source for any increase in vascular markers. This may be of particular importance in AR (Fig. 4)(3, 4). Identification of other potential sources of fHb in normal and NAR subjects requires further investigation.

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