Comparison of Two Methods of Processing Induced Sputum: Selected versus Entire Sputum

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Sputum analysis is increasingly used to assess airway inflammation in asthma. The analysis of sputum is currently performed with two techniques, i.e., analysis of selected sputum (plugs) and analysis of entire sputum. To investigate the diagnostic value of these two methods, we compared total and differential cell counts and supernatant eosinophil cationic protein (ECP) in selected and entire sputum collected on two occasions in a group of healthy and asthmatic subjects. We induced sputum with hypertonic saline in 18 asthmatics and in eight healthy subjects. On one occasion we analyzed selected sputum, and on another occasion we analyzed entire sputum. In each sample we measured total and differential cell counts and ECP concentration in supernatant. We found a higher percentage of eosinophils (15.3 versus 8.3%; $p < 0.01$), more viable nonsquamous cells (80.6 versus 71.8%; $p < 0.01$), and higher levels of ECP (548 versus 105 μg/L; $p < 0.001$) in selected sputum as compared with entire sputum, whereas the percentage of neutrophils was higher in the entire sputum (42.7 versus 33.3%; $p < 0.05$). The percentage of eosinophils and ECP concentration were significantly and similarly increased in both selected and entire sputum of asthmatic subjects, i.e., independent of the method of sputum analysis. In conclusion, the selected sputum method may indeed provide more viable cells, more eosinophils, and a higher concentration of ECP. However, both the selected sputum and the entire sputum method have the same diagnostic value in distinguishing asthmatics from healthy subjects. Spanevello A, Beghé B, Bianchi A, Migliori GB, Ambrosetti M, Neri M, Ind PW. Comparison of two methods of processing induced sputum: selected versus entire sputum.

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Sputum analysis is increasingly used to assess airway inflammation in asthma (1, 2). The analysis of sputum is currently performed with two techniques, i.e., analysis of selected sputum and analysis of entire sputum. The first involves collecting and analyzing the more viscid portions of mucus (plugs) extracted from entire sputum as described by Popov and colleagues (2). The second involves collecting and analyzing the entire sputum, including saliva, as described by Fahy and colleagues (3). Both techniques have been shown to be valid, i.e., to distinguish between asthma and normal subjects, to detect acute airway inflammation during allergen challenge, and to detect reduced airway inflammation after treatment (1–6), and reproducible (7–9).

To measure the diagnostic value of these two techniques, in this study we compared these two methods of analysis of sputum by measuring total and differential cell counts and supernatant eosinophil cationic protein (ECP) in induced sputum collected in normal and in asthmatic subjects. To our knowledge this is the first study comparing total and differential cell counts and ECP levels in induced sputum collected on two occasions in the same subjects using two different techniques of processing samples.

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METHODS

Subjects

We studied 18 subjects with bronchial asthma and eight healthy subjects (Table 1). Asthma was defined as a clinical history of intermittent wheeze, cough, chest tightness, or dyspnea, and documented reversible airflow limitation with an improvement in $FEV_1 \geq 20\%$ after albuterol (200 μg) when FEV_1 was $\leq 70\%$ predicted or methacholine airway responsiveness ($PD_{20} \geq 1{,}600$ μg) when FEV_1 was $\geq 70\%$ (10). All subjects were stable as demonstrated by the low daily variability ($< 15\%$) of peak flow measurements during 2 wk before the study (11). Medications were unchanged for at least 1 mo before the study, except for inhaled β_2-agonists taken as required. None referred a history of an upper respiratory infection in the preceding four wk.

Healthy subjects were nonsmokers with no history of asthma or other respiratory symptoms, $FEV_1 > 80\%$ predicted, and methacholine airway responsiveness $PD_{20} > 1{,}600$ μg. The study was approved by the Ethics Committee of Fondazione Salvatore Maugeri, and all subjects gave written informed consent.

Study Design

Subjects attended the laboratory on 3 d at the same time of the day. On the first visit consent was obtained, subject characteristics were doc-

TABLE 1

CHARACTERISTICS OF SUBJECTS

	Asthmatics	Healthy Subjects
Subjects, n	18	8
Age, yr*	41 ± 17	42 ± 17
Sex, male/female	8/10	3/5
Smoking, (ex)	1 (3)	0 (2)
Atopy†	12	2
FEV_1, % pred*	91 ± 21‡	119 ± 16
FVC, % pred*	103 ± 16	112 ± 14
PD_{20} FEV_1, μg§	220	> 1,600
Inhaled steroid therapy	3	–

* Values are mean ± SD.
† Defined as one or more positive allergy skin prick tests.
‡ $p < 0.001$, significantly different.
§ Methacholine PD_{20} geometric mean.

umented by questionnaire, and skin prick tests and spirometry (Vitalograph, Buckinghamshire, UK) were performed. If spirometry showed $FEV_1 \geq 70\%$ a methacholine inhalation test was carried out, and the results were expressed as the PD_{20} in noncumulative units; if $FEV_1 \leq 70\%$, reversibility of airway obstruction was performed using 200 μg inhaled albuterol. On the second and third visits (24 h apart) the sputum was induced after inhaled albuterol (200 μg) and was analyzed using two different methods: the selected sputum and the entire sputum. Before the second visit the order in which the two methods were performed was randomized in each subject. In a subset of eight asthmatics sputum was induced on two other occasions, 24 h apart, within 2 wk after the end of the study. The samples were analyzed with the same technique (selected sputum) to show any possible influence of the first inhalation of hypertonic saline on the cellularity of the second analysis.

Sputum Induction

FEV_1 and FVC were measured before and 10 min after albuterol inhalation (two puffs; 200 μg) and subjects then inhaled hypertonic (4.5%) saline nebulized for increasing time periods, 1, 2, 4, 8, and 16 min, FEV_1 was repeated 1 min after each inhalation period (12). Saline solutions were nebulized by an ultrasonic nebulizer (DeVilbiss 65; DeVilbiss Corp., Somerset, PA).

Collected sputum and saliva samples were examined within 2 h after the 16-min inhalation. The duration of inhalation of hypertonic saline was the same (five inhalation times of 1 to 16 min) in each subject on the two occasions.

Selected sputum. Sputum was collected and processed using methods identical to those described by Popov and colleagues (2). Selected portions (plugs) of the sputum sample originating from the lower respiratory tract were chosen using an inverted microscope and weighed. The residual portion was placed in a tube and the volume was recorded. Both portions were treated with dithiotreitol (DTT) (Sputolysin; Calbiochem Corp., San Diego, CA), freshly prepared in a dilution of one in 10 with distilled water. The selected portion was treated with a volume (in microliters) equal to two times the weight of the sputum portion (in milligrams), whereas the residual portion was diluted by a volume of DTT equal to the volume recorded.

The portions were placed in a shaking water bath at 37° C for 20 min to ensure complete homogenization. The liquid resulted from selected portions was further diluted with PBS in a volume equal to the sputum plus DTT. The suspensions were filtered through nylon gauze to remove mucus and were centrifuged at 1,000 *g* for 10 min. The supernatants were aspirated and frozen at −70° C for later ECP (μg/L) analysis by radioimmunoassay (RIA; Kaby Pharmacia Diagnostic AB, Uppsala, Sweden). The cell pellets were resuspended in PBS. Total cell count (TCC) and viability (Trypan blue exclusion method) were determined using a Burkers chamber hemocytometer. Cell suspensions were placed in a CYTO-TEK centrifuge (Miles Scientific, Milan, Italy), and cytospins were prepared at 500 rpm for 6 min. Cytospin slides were stained with Diff-Quik for overall differential cell count on 500 (selected sputum) and 200 (residual portion) nucleated nonsquamous cells by two examiners. All sputum counts and measurements were performed blind to the clinical details. Definition of an adequate selected sputum was one in which there were fewer than 20% squamous cells and viability > 50%.

Entire sputum. Sputum was collected and the saliva samples were processed using method identical to those described by Fahy and colleagues (3). In brief, the volume of induced sputum sample and the samples of saliva were determined, and an equal volume of DTT (Sputolysin; Calbiochem Corp.), freshly prepared in a dilution of one in 10 with distilled water, was added. The samples were then placed in a shaking water bath at 37° C for 20 min to ensure complete homogenization. At this point the processing was similar to that used for the residual portion. Definition of an adequate induced sputum sample was one in which there were fewer than 80% squamous cells on differential cell count.

Statistical Analysis

Descriptive statistics were used to summarize clinical and demographic characteristics of the subjects. Comparison of eosinophils and ECP in selected and entire sputum are graphically reported as proposed by Bland and Altman (13). Grouped data were reported as

TABLE 2

CELL VIABILITY (%), TOTAL CELL COUNT (× 10^5/ml), DIFFERENTIAL CELL COUNT (%), AND ECP (μg/L) IN SELECTED SPUTUM, ENTIRE SPUTUM, AND RESIDUAL PORTION IN ALL SUBJECTS, HEALTHY SUBJECTS, AND ASTHMATICS*

	SS All Subjects	ES All Subjects	RP All Subjects	SS Healthy Subjects	ES Healthy Subjects	RP Healthy Subjects	SS Asthmatics	ES Asthmatics	RP Asthmatics
Viability, %	80.6 ± 1.6‡¶	71.8 ± 1.9	70.2 ± 2.0	83.0 ± 1.9	73.0 ± 5.1	69.3 ± 4.6	79.5 ± 2.1‡¶	71.2 ± 1.8	70.6 ± 2.2
TCC, × 10^5/ml	16.5 ± 3.2¶	11.1 ± 0.9	6.1 ± 0.9	13.5 ± 5.0‖	10.9 ± 1.5	4.8 ± 1.6	18.1 ± 4.6¶	11.2 ± 1.1	6.6 ± 1.0
Macro, %	49.9 ± 4.8	46.2 ± 4.1	48.2 ± 4.0	62.6 ± 8.1	58.0 ± 7.7	55.3 ± 8.2	44.3 ± 5.6	40.9 ± 4.4	45.1 ± 4.4
Neutro, %	33.3 ± 4.7†‖	42.7 ± 4.3	41.2 ± 4.4	34.7 ± 8.1	38.0 ± 8.3	38.6 ± 9.4	32.6 ± 5.9†	44.9 ± 5.1	42.3 ± 5.0
Eos, %	15.3 ± 4.7†¶	8.3 ± 3.0	7.7 ± 3.0	0.8 ± 0.3	0.6 ± 0.3	0.6 ± 0.2	21.8 ± 6.2‡¶	11.8 ± 4.2	10.9 ± 4.1
Lympho, %	0.6 ± 0.2	0.6 ± 0.2	0.3 ± 0.1	0.4 ± 0.4	0.4 ± 0.3	0.4 ± 0.2	0.8 ± 0.2	0.7 ± 0.3	0.3 ± 0.1
Epi cells, %	0.9 ± 0.3	2.2 ± 0.8	2.6 ± 1.0	1.6 ± 0.9	3.2 ± 2.0	5.2 ± 3.1	0.5 ± 0.3	1.7 ± 0.7	1.4 ± 0.5
Sq cells, %	2.7 ± 0.6§**	55.6 ± 3.1	57.0 ± 3.2	2.9 ± 0.8‡¶	62.2 ± 3.4	59.3 ± 5.8	2.6 ± 0.8§**	54.3 ± 4.3	56.1 ± 3.9
ECP, μg/L	548 ± 101§**	105 ± 20	74 ± 13	180 ± 55	40 ± 10	28 ± 3.3	720 ± 123§**	133 ± 2.7	91 ± 17

Definition of abbreviations: SS = selected sputum; ES = entire sputum; RP = residual portion; TCC = total cell count; Macro = macrophages; Neutro = neutrophils; Eos = eosinophils, Lympho = lymphocytes; Epi cells = epithelial cells; Sq cells = squamous cells; ECP = eosinophil cationic protein.

* Data are expressed as mean and SEM.
† < 0.05, significantly different from entire sputum.
‡ < 0.01, significantly different from entire sputum.
§ < 0.001, significantly different from entire sputum.
‖ $p < 0.05$, significantly different from residual portion.
¶ $p < 0.01$, significantly different from residual portion.
** $p < 0.001$, significantly different from residual portion.

TABLE 3

EOSINOPHILS (%) AND ECP (μg/L) IN SELECTED SPUTUM AND IN ENTIRE SPUTUM

Asthmatics	Eos (*SS*)	Eos (*ES*)	ECP (*SS*)	ECP (*ES*)	Healthy Subjects	Eos (*SS*)	Eos (*ES*)	ECP (*SS*)	ECP (*ES*)
1	45	44	ND†	ND	1	0	0	ND	ND
2	75	69	ND	ND	2	0	0	12	60
3	4	4	947	180	3	0	0	9	14
4	39	14	1,790	169	4	1	0	185	28
5	8	3	1,151	128	5	2	2	428	74
6	1	1	73	116	6	1	1	150	67
7	5	8	293	48	7	0	0	230	14
8	36	9	1,176	78	8	2	1	248	20
9	25	12	448	136					
10	15	4	1,086	400					
11	2	1	1,022	314					
12	0	0	39	39					
13	86	20	ND	ND					
14	14	8	440	89					
15	34	12	800	161					
16	0	1	456	91					
17	0	1	592	13					
18	2	0	492	39					
Mean	21.8*	11.8*	720*	133*		0.8	0.6	180	40
SEM	6.2	4.2	123	27		0.3	0.3	55	10

For definition of abbreviations, *see* Table 2.

* Refers to a significant difference from healthy subjects sputum, $p < 0.01$.

† Not done.

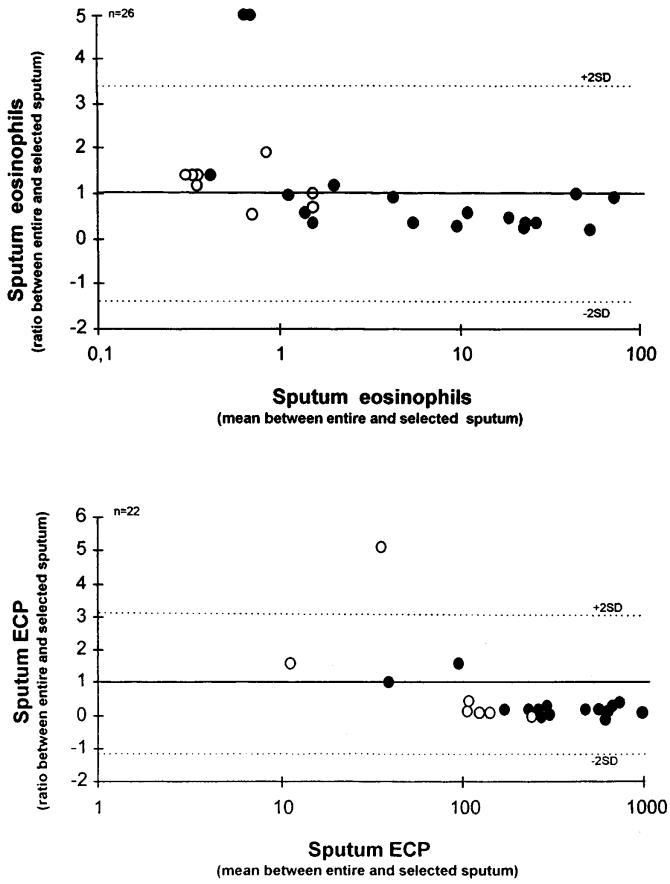

Figure 1. Bland and Altman plot of eosinophils percentage and ECP concentration as mean of two values obtained with two different methods (entire and selected sputum) compared with the ratio of the value obtained with the selected and entire method. Asthmatics (*closed circles*) and healthy subjects (*open circles*). SD is standard deviation. *n* is the number of measures plotted.

arithmetic mean and standard error of the mean (SEM). Based on data distribution nonparametric tests were used. Differences between selected sputum, entire sputum, and residual portion were assessed by Wilcoxon's signed rank test. For data normally distributed (e.g., macrophages, neutrophils) differences were assessed by the two-tailed paired *t*-test. The comparison between groups (asthmatics versus healthy subjects) was assessed by the Mann-Whitney U test, $p < 0.05$ was considered statistically significant.

RESULTS

The comparisons between selected sputum, entire sputum, and the residual portion for cell viability (%), total cell count ($\times 10^5$/ml), differential cell count (%), and ECP (μg/L) are presented in Table 2. In brief, the selected sputum contained significantly higher cell viability, percentage of eosinophils, and ECP concentration with respect to entire sputum and residual portion considering both all subjects or asthmatics only. The entire expectorate and the residual portion showed a significant higher percentage of neutrophils and squamous cells. In saliva samples, squamous cells were found to constitute 99 ± 0.7% of total cells.

With both methods of the selected and entire sputum eosinophils and ECP were found to be significantly higher in asthmatics than in healthy subjects (Table 3). Comparison of eosinophils and ECP in selected and entire sputum are graphically reported as proposed by Bland and Altman (Figure 1).

No significant differences for total cells (25.2 ± 5 versus 23.8 ± 7 $\times 10^5$/ml), ECP concentration (402 ± 75 versus 431 ± 69 μg/L), and any cell type were found comparing the two samples obtained 24 h apart using the same technique (selected sputum) in eight asthmatic subjects (Figure 2).

DISCUSSION

In this study we observed that, as compared with entire sputum, the analysis of selected portions of sputum resulted in an increased number of eosinophils, viable cells, and in increased levels of supernatant ECP. By contrast, the percentage of neutrophils was higher in the entire sputum. However, we also observed that both methods of analysis have the same diagnostic value in distinguishing asthmatics from healthy subjects. To our knowledge this is the first study that has compared the total and differential cell counts and the levels of ECP in induced sputum collected on two occasions from asthmatic and healthy subjects, using two different techniques of processing samples performed in the same group of subjects.

The increased percentage of eosinophils and the higher concentration of ECP in selected sputum with respect to the

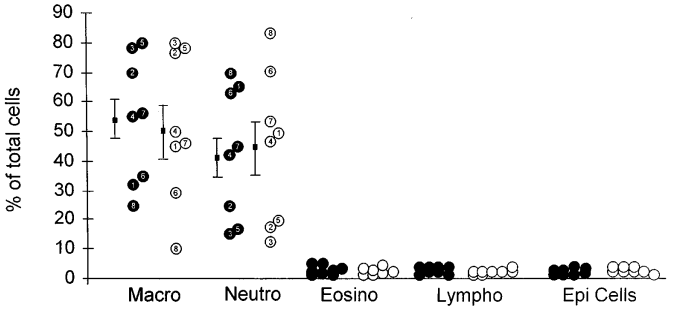

Figure 2. Scattegram of cell counts in two selected sputum repeated 24 h apart in eight asthmatic subjects (1–8). First day (*closed circles*), second day (*open circles*). Macrophages (Macro), Neutrophils (Neutro), Eosinophils (Eosino), Lymphocytes (Lympho), and Epithelial cells (Epi Cells). Mean and SEM are reported for macrophages and neutrophils.

entire sputum and residual part may be explained by the tendency of eosinophils to be sequestred in the more viscid portions of the sputum samples. However, the presence of eosinophils in the residual part also indicates that if a good selection of plugs has been done (2% of squamous cells in selected sputum) some inflammatory cells remain in the residual portion of the sample, as previously described (14).

Compared with selected sputum the percentage of neutrophils was higher in the entire sputum. This is unlikely due to salivary contamination as squamous cells are about 99% of the cells present in saliva. More likely, the higher percentage of eosinophils in the selected portion of sputum explain the lower percentage of neutrophils. If this was the case, "selected sputum" might underestimate the percentage of neutrophils in airway secretion of asthmatics, and this should be considered, particularly for those forms of asthma mainly associated with sputum neutrophils (e.g., asthma exacerbations caused by viral infections of the upper airways).

We also found an increased viability of nonsquamous cells in selected sputum, which might be explained by the capacity of the mucus to protect the cells from DTT or salivary enzymes (15, 16).

Apart from the differences mentioned above between the selected portion and entire sputum, an important finding of this study is that both techniques were able to distinguish asthmatics from healthy subjects in the same group of subjects. Although this data confirms previous reports (1–3, 8), we believe that the conclusions of our study are stronger because the diagnostic value of both methods was demonstrated in the same group of subjects.

In conclusion, although the selected sputum might underestimate the percentage of neutrophils, it is a better method of analyzing induced sputum, providing more viable cells, more eosinophils, and higher concentration of ECP in asthmatics. However, both the selected sputum and the entire sputum method have the same ability to distinguish asthmatics from healthy subjects.

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References

1. Pin, I., P. G. Gibson, R. Kolendovicz, A. Girgis-Gabardo, J. Denburg, F. E. Hargreave, and J. Dolovich. 1992. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax* 47:25–29.
2. Popov, T., M. M. M. Pizzichini, E. Pizzichini, R. Kolendowicz, Z. Punthakee, J. Dolovich, and F. E. Hargreave. 1995. Some technical factors influencing the induction of sputum for cell analysis. *Eur. Respir. J.* 8:559–565.
3. Fahy, J. V., J. Liu, H. Wong, and H. A. Boushey. 1993. Cellular and biochemical analysis of induced sputum from asthmatic and from healthy subjects. *Am. Rev. Respir. Dis.* 147:1126–1131.
4. Pin, I., A. P. Freitag, P. M. O'Byrne, A. Girgis-Gabardo, R. M. Watson, J. Dolovich, J. A. Denburg, and F. E. Hargreave. 1992. Changes in the cellular profile of induced sputum after allergen-induced asthmatic responses. *Am. Rev. Respir. Dis.* 145:1265–1269.
5. Claman, D. M., H. A. Boushey, J. Liu, H. Wong, and J. V. Fahy. 1994. Analysis of induced sputum to examine the effects of prednisone on airway inflammation. *J. Allergy Clin. Immunol.* 94:861–869.
6. Fahy, J. V., H. Wong, J. Liu, and H. A. Boushey. 1994. Analysis of cellular and biochemical constituents of induced sputum after allergen challenge: a method for studying allergic airway inflammation. *J. Allergy Clin. Immunol.* 93:1031–1039.
7. Pizzichini, E., M. M. M. Pizzichini, A. Efthimiadis, S. Evans, M. M. Morris, D. Squillace, G. J. Gleich, J. Dolovich, and F. E. Hargreave. 1996. Indices of airway inflammation in induced sputum: reproducibility and validity of cell fluid-phase measurements. *Am. J. Respir. Crit. Care Med.* 154:308–317.
8. Spanevello, A., G. B. Migliori, A. M. Sharara, L. Ballardini, P. Bridge, P. Pisati, M. Neri, and P. W. Ind. 1997. Induced sputum to assess airway inflammation: a study of reproducibility. *Clin. Exp. Allergy* (In press)
9. In't Veen, J. C. C. M., H. W. F. M. De Gouw, H. H. Smits, J. K. Sont, P. S. Hiemstra, P. J. Sterk, and E. H. Bel. 1996. Repeatibility of cellular and soluble markers of inflammation in induced sputum from patients with asthma. *Eur. Respir. J.* 9:2441–2447.
10. Quanjer, P. H., G. J. Tammeling, J. E. Cotes, R. Pedersen, R. Peslin, and J. C. Yernault. 1993. Lung volumes and forced ventilatroy flows: report of working party, standardization of lung function tests. European Community for Steel and Coal. *Eur. Respir. J.* 6(Suppl. 16):5–40.
11. National Heart, Lung, and Blood Institute. 1995. Global strategy for asthma management and prevention. National Institutes of Health, Bethesda, MD. NHLBI Publication No. 95-3659.
12. Iredale, M. J., S. A. R. Wanklin, I. P. Phillips, T. Krausz, and P. W. Ind. 1994. Noninvasive assessment of bronchial inflammation in asthma: no correlation between eosinophilia of induced sputum and bronchial responsiveness to inhaled hypertonic saline. *Clin. Exp. Allergy* 24:940–945.
13. Bland, J. M., and D. G. Altman. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* i:307–310.
14. Pizzichini, E., M. M. M. Pizzichini, A. Efthimiadis, F. E. Hargreave, and J. Dolovich. 1996. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. *Eur. Respir. J.* 9:1174–1180.
15. Hansel, T. T. 1994. The cardinal importance of sputum microscopy. *Clin. Exp. Allergy* 24:695–697.
16. Tang, C. S., I. T. M. Kung. 1993. Homogenization of sputum with dithiothreitol for early diagnosis of pulmonary malignancies. *Acta Cytol.* 37:689–693.