Bronchoalveolar Lavage Cellularity

The Distribution in Normal Volunteers[1-3]

ROBERT K. MERCHANT,[4] DAVID A. SCHWARTZ,[5] RICHARD A. HELMERS, CHARLES S. DAYTON, and GARY W. HUNNINGHAKE

SUMMARY Bronchoalveolar lavage (BAL) is used to obtain inflammatory cells from the lung. For clinical research, parametric statistics are frequently used to compare cells present in BAL of patients with lung disease with cells present in BAL of normal subjects. To determine if these populations can be compared in this manner we performed BAL on 111 never-smoking, normal volunteers and determined: (1) the mean, median, standard deviation, and range of the cells in BAL; (2) whether the data are normally distributed and satisfy the criteria for use of parametric statistical analysis. The BAL cellularity was expressed as a percentage of total cells, cells per milliliter return, and total cells per lavage. Regardless of the means of expression, no measure of BAL cellularity (total cells, macrophages, lymphocytes, neutrophils, or eosinophils) conformed to the normal (bell-shaped) distribution when tested for goodness of fit with the G statistic (all $p < 0.001$). The lack of fit to the normal distribution was not substantially altered by either the method of expressing the data (i.e., cells per milliliter, total cells, or percent of cells) or log transformation of the data. The poor fit in all cases resulted from clumping of the data about the mean and large tails. The percent of cells were, therefore, tested for goodness of fit to the Poisson distribution, a distribution of discrete variables. The neutrophil and eosinophil percentages resulted in an excellent fit to the Poisson distribution, but macrophage and lymphocyte percentages did not. The observations that the BAL data are not normally distributed indicate that nonparametric statistics should be used for comparing BAL cell data between normal subjects and patients with lung disease.

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Introduction

Bronchoalveolar lavage (BAL) is used as both a research and a clinical tool (1-5). The technique permits sampling of inflammatory cells and proteins in the distal airways and alveoli, and it is associated with minimal risk (6, 7). In patients with a variety of lung diseases, the numbers and types of cells present in BAL fluid differ from those present in BAL from normal subjects, suggesting that BAL may be of value in the diagnosis and management of these diseases (1, 2, 8). In order to compare results of BAL between patients with lung disease and normal subjects, however, it is necessary to be certain that the underlying distribution justifies the specific methods of statistical analysis.

All BAL studies of normal volunteers reported in the literature to date are relatively small in size. Because of this limitation, the distribution of cells in BAL fluid in the control populations is not well defined. Thus, it is difficult to compare the BAL cell content from these control populations with either individual patients or with study subjects with lung disease. For example, important aspects of the distributions of BAL cell counts include not only the average value (mean) and the amount of variation (standard deviation) but also the randomness, or independence, of the samples and whether or not the cell counts conform to a normal distribution. Relatively small samples may give a reasonable estimate of the mean, but they are less reliable in determining the nature of the tails of the distribution.

To determine the nature of the distribution of cells in BAL fluid, we evaluated BAL data from a large number of normal volunteers. To identify whether the distribution was substantially altered by the method of expressing the data, we compared three different methods of presentation of the cell counts (cells per milliliter return, cells/100 ml of infusate, and percent of total cells). Moreover, we determined whether the data were normally distributed and satisfied the criteria established for parametric statistical analyses. In addition, the amount of variation within normal subjects was assessed in 27 who underwent serial lavages.

Methods

Study Population

One hundred eleven normal nonsmoking volunteers underwent 166 bronchoalveolar lavages. Twenty-seven of the 111 subjects had at least two lavages. The mean age of the volunteers was 30 yr with a range of 20 to 48 yr (figure 1). Sixty-three percent were male and 37% were female. For the purposes of this study, nonsmokers were considered to be lifetime never-smokers (< 20 cigarettes in their lifetime). None of the normal volunteers had any evidence on history and physical examination of respiratory disease, including asthma, allergic rhinitis, interstitial lung disease, exposure to pneumoconiotic dust, or recent viral illness. In addition to the 111 nonsmoking volunteers, 19 otherwise healthy current smoking volunteers were also lavaged. Although this sample of smokers was small, we included these volunteers to investigate the effect of cigarette smoking on BAL cellularity. For subjects who underwent more than one BAL, only the first lavage was included in

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[1] From the Pulmonary Disease Division, Department of Internal Medicine, Department of Veterans Affairs Medicinal Center, College of Medicine, The University of Iowa, Iowa City, Iowa.

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[3] Correspondence and requests for reprints should be addressed to Gary W. Hunninghake, M.D., Department of Internal Medicine, College of Medicine, The University of Iowa, Iowa City, IA 52242.

[4] Recipient of Clinical Investigator Award ES-00196 from the National Institute of Environmental Health Sciences.

[5] Recipient of Clinical Investigator Award ES-00203 from the National Institute of Environmental Health Sciences.

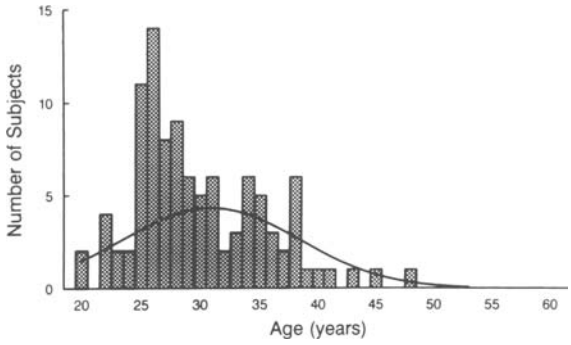

Fig. 1. Age distribution of the study population. The expected normal distribution (based on the mean of 29.9 and standard deviation of 5.5) is superimposed on the histogram. Median 28.0; mean 29.9; SD 5.5.

TABLE 1

EFFECT OF NUMBER OF SEGMENTS LAVAGED ON PERCENT RETURN AND CELLS/ML RETURN*

Lavaged Segment No.	Segments Lavaged (*n*)	Return (%)	Cell Return (× 10^4/ml)
1	8	63.7 ± 7.1	14.9 ± 5.4
2	16	67.0 ± 10.3	15.0 ± 7.2
3	25	70.3 ± 11.5	12.0 ± 4.6
4	68	81.5 ± 16.9	12.1 ± 10.6

* Values are mean ± standard deviation.

the comparisons. The subsequent lavages were used to examine the variation within normal subjects from one lavage to the next. The study was approved by the Human Use Committee at The University of Iowa.

Bronchoalveolar Lavage

The subjects underwent fiberoptic bronchoscopy and BAL as previously described (9). Lavage was carried out in the lingula and/or the right middle lobe. Five 20-ml aliquots (for a total of 100 ml) of normal saline at 37° C were instilled into each segment lavaged. The number of segments lavaged ranged from one to four. When more than one segment was lavaged, the results from the segments were averaged. The lavage samples were processed as previously described (9). The total cell count was determined using a hemocytometer. The percentage of each cell type was determined using a Wright-Giemsa-stained cytocentrifuge preparation.

Statistics

The cell counts were displayed as frequency distributions expressed as cells per milliliter BAL fluid recovered (cells/ml), cells per 100 ml of infused BAL fluid (total cells), and as percentage of the total cell population. We evaluated whether these different methods used to express BAL cell counts altered the underlying distribution and whether the results were normally distributed (an assumption implicit in parametric statistical test). The results were expressed as the mean and the standard deviation. The mean and median are reported for each set of data since the difference between the mean and the median provides an estimate of the skew of the distribution. The goodness of fit to the normal distribution was statistically assessed by using the Kolomogorof-Smirnoff test and visually assessed by superimposing the normal distribution over the observed distribution. For variables that could be considered discrete (i.e., the percent of total cells), the Kolomogorof-Smirnoff test was also used to test the goodness of fit to a Poisson distribution. The Poisson distribution differs from the normal distribution in that it is a distribution of the frequency of discrete variables in contrast to continuous variables.

The data were analyzed for goodness of fit both before and after log transformation. For the purpose of log transformation, the zero values were converted to a positive value just below the limit of detection of the assay.

For the 27 volunteers with serial lavages, we examined the differences in the values obtained between the first and second lavage. We then plotted the distribution of these differences to examine the range of the differences and also to determine if this distribution approximated a normal distribution. The relationship between the first and second lavage was examined using Spearman's rank correlation and graphic comparison.

When comparing the BAL cell counts from nonsmoking volunteers and smoking volunteers we used nonparametric statistics (10). This approach is supported by the nonnormal distribution of the BAL cell counts demonstrated in the nonsmoking volunteers.

Results

BAL was well tolerated by all the subjects. There were no significant complications. The number of segments lavaged had a minimal effect on the percent return (volume returned/volume infused × 100) or the cells/ml return (table 1). Therefore, for subjects who had more than one segment lavaged, the data were normalized to a single 100-ml lavage by dividing the total cells returned by the number of segments lavaged.

For the nonsmoking volunteers, the cells/ml return ranged from 2 to 84 × 10^4 (figure 2). Only five subjects (4.5%) had more than 25 × 10^4 cells/ml. The mean cells/ml was 12.7 ± 9.1 × 10^4, with a median of 11 × 10^4. The mean cells/100 ml infused was 9.5 ± 8.3 × 10^6, with a median of 8 × 10^6 and a range of 1.7 to 83 × 10^6.

As expected, the majority of cells were macrophages. Only four subjects (3.6%) had less than 80% macrophages (figure 3). The percent of macrophages ranged from 100 to 61%, with a mean of 93.2 ± 5.8% and a median of 95%. Expressed as cells/ml return, the mean was 12.1 ± 10 × 10^4, with a range of 2.3 to 80.4 × 10^4 and a median of 9.9 × 10^4. The macrophages/100 ml infused ranged from 1.6 to 80.4 × 10^6, with a mean of 8.9 ± 7.9 × 10^6 and a median of 7.68 × 10^6.

Lymphocytes were the next most frequent cell type, with a mean of 6.1 ± 5.6% and a median of 5% (figure 4). Four subjects had 0% lymphocytes, and three (2.7%) had more than 15%. The highest observed lymphocyte percentage was 38%. The lymphocytes/ml return ranged from zero to 6.0 × 10^4, with a mean of 0.8 × 10^4 and a median of 0.5 × 10^4. The mean lymphocytes/100 ml infused was 0.57 ± 0.66 × 10^6, with a range of zero to 4.28 × 10^6 and a median of 0.34 × 10^6.

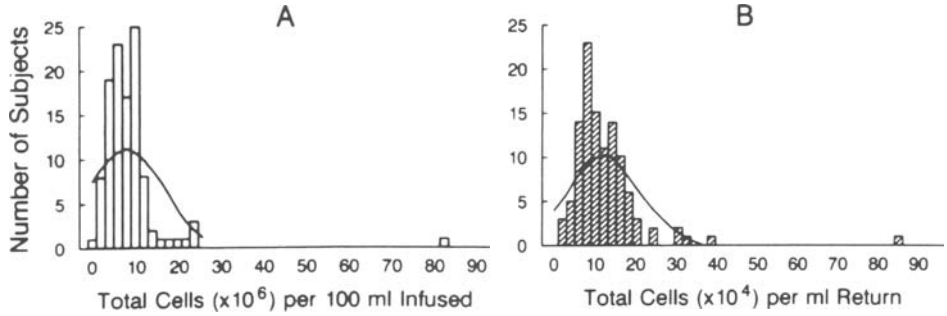

Fig. 2. Distribution of the total cells obtained by BAL. Panel A shows the distribution of total cells per 100 ml infused. Median 8.0; mean 9.5; SD 8.3. Panel B shows the distribution of total cells per ml return. The observed distributions are significantly different ($p < 0.001$) from the normal distribution, which is superimposed. Median 11.0; mean 12.7; SD 9.1.

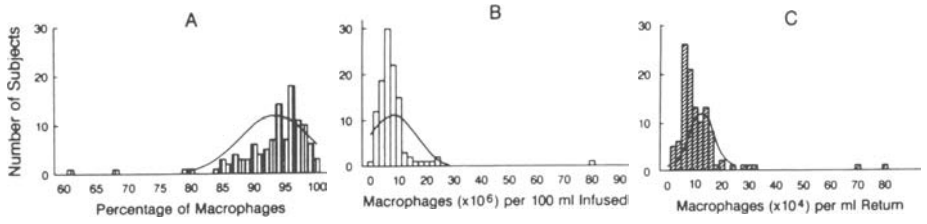

Fig. 3. Distribution of macrophages obtained by BAL. In Panel A, macrophages are expressed as a percentage of the total cells. Median 95.0; mean 93.2; SD 5.8. Panel B depicts macrophages per 100 ml infused. Median 7.68; mean 8.9; SD 7.9. Panel C shows macrophages per ml return. Median 9.9; mean 12.6; SD 9.1. In each case the observed distributions are significantly different ($p < 0.001$ for each) from the normal distribution, which is superimposed.

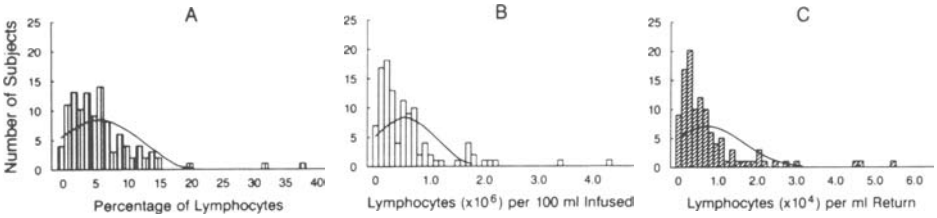

Fig. 4. Distribution of lymphocytes obtained by BAL. In Panel A, lymphocytes are expressed as a percentage of the total cells. Median 5.0; mean 6.1; SD 5.6. Panel B depicts lymphocytes per 100 ml infused. Median 0.34; mean 0.57; SD 1.01. Panel C shows lymphocytes per ml return. Median 0.50; mean 0.81; SD 1.01. In each case the observed distributions are significantly different ($p < 0.001$ for each) from the normal distribution, which is superimposed.

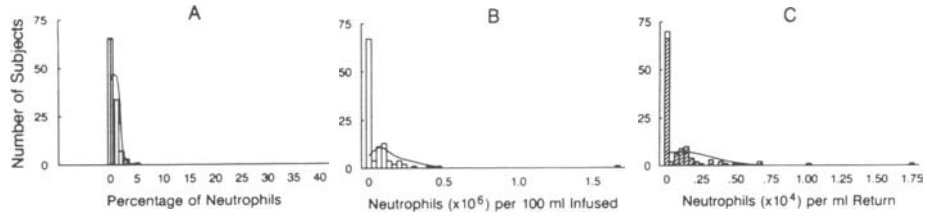

Fig. 5. Distribution of neutrophils obtained by BAL. In Panel A, neutrophils are expressed as a percentage of the total cells. Median 0.00; mean 0.54; SD 0.83. Panel B depicts neutrophils per 100 ml infused. Median 0.00; mean 0.06; SD 0.18. Panel C shows neutrophils per ml return. Median 0.00; mean 0.08; SD 0.20. In each case the observed distributions are significantly different ($p < 0.001$ for each) from the normal distribution, which is superimposed.

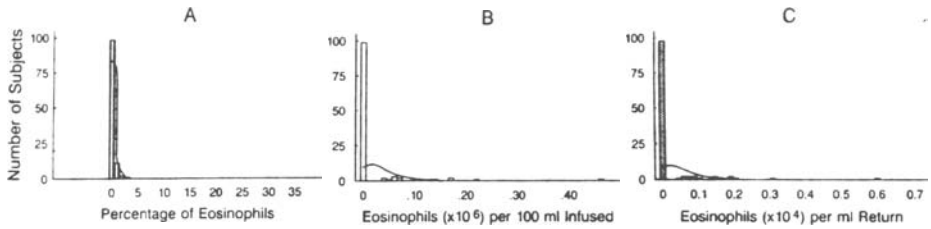

Fig. 6. Distribution of eosinophils obtained by BAL. In Panel A, eosinophils are expressed as a percentage of the total cells. Median 0.00; mean 0.14; SD 0.44. Panel B depicts eosinophils per 100 ml infused. Median 0.00; mean 0.02; SD 0.06. Panel C shows eosinophils per ml return. Median 0.00; mean 0.02; SD 0.06. In each case the observed distributions are significantly different ($p < 0.001$ for each) from the normal distribution, which is superimposed.

Neutrophils were not observed in the majority of subjects, but they were present in 45 (41%) (figure 5). The mean percentage was 0.54 ± 0.83%, and the median was 0%. The highest neutrophil percent observed in this population was 5%. Neutrophils/ml return ranged from zero to 1.68 × 10^4, with a mean of 0.08 ± 0.20 × 10^4 and a median of zero. The range of neutrophils/100 ml infused was zero to 1.68 × 10^6, with a mean of 0.06 ± 0.18 × 10^6 and a median of zero.

Eosinophils were the least frequently observed cell type, present in only 14 (13%) (figure 6). None of the subjects had more than 3% eosinophils, and the mean percent eosinophils was 0.15 ± 0.47%; the median was zero. The mean eosinophils/ml return was 0.02 ± 0.08 × 10^4, with a range of zero to 0.63 × 10^4 and a median of zero. Eosinophils/100 ml infused ranged from zero to 0.46 × 10^6, with a mean of 0.02 ± 0.06 × 10^6 and a median of zero.

The pattern of the distributions varied greatly for the different cell types (figures 3 to 6). Within each cell type, the pattern of the distribution was minimally affected by expressing the values as either cells per 100 ml infused, cells per milliliter return, or as a percentage (figures 3 to 6). None of the distributions conformed to the expected normal distribution. This can be seen by visual inspection of the observed histograms with the superimposed line representing the normal distribution. This observation was statistically confirmed by the G test for goodness of fit, which gave a $p = 0.001$ for each distribution when tested against the normal distribution. This indicates that none of these distributions satisfied the statistical requirements of the normal distribution. Visual inspection of the histograms reveals that the deviation from the normal distribution results from clumping, with an excess of samples near the mean and long tail(s) beyond the limits of the normal distributions.

Log transformation of the data resulted in normalization of the macrophage and lymphocyte distributions when the counts were expressed as cells/ml return or cells/100 ml infused (macrophages/ml return, $p = 0.50$; macrophages/100 ml return, $p = 0.41$; lymphocytes/ml return, $p = 0.34$; lymphocytes/100 ml infused, $p = 0.39$). Log transformation of macrophage and lymphocyte percentages failed to normalize the distributions ($p < 0.001$ for each). Neutrophil and eosinophil distributions were not normalized by log transformation regardless of the mode of expression ($p < 0.001$ for each).

The percentages of total cells for each cell type can be evaluated as discrete variables. To determine if a better fit would occur using a frequency distribution for discrete variables, the percent cell distributions were modeled to the Poisson distribution. The percentages of neutrophils and of eosinophils showed an excellent fit to the Poisson distribution ($p > 0.5$ for each). The percent of lymphocytes and of macrophages did not fit this distribution ($p = 0.001$ for each).

The influence of age on cellularity was assessed using Spearman's coefficient of rank correlation. Age was weakly cor-

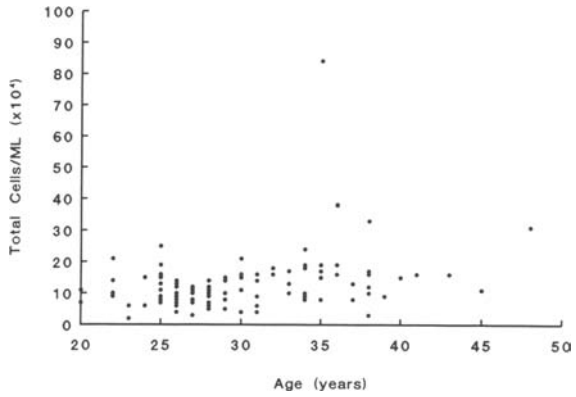

Fig. 7. The effect of age on total cellularity of BAL return (expressed as cells/ml return).

TABLE 2

SPEARMAN'S RANK CORRELATION BETWEEN SERIAL LAVAGES IN THE SAME SUBJECT (n = 27)

	r	p Value
Cells/ml × 10^4	0.67	< 0.001
Macrophages, %	0.035	0.861
Lymphocytes, %	0.031	0.877
Neutrophils, %	0.284	0.151
Eosinophils, %	−0.100	0.620

related with the total cellularity, but the correlation was highly significant (r = 0.31, p = 0.001). There was no correlation, however, with the percentages of the cell types (percent macrophages: r = 0.09, p = 0.38; percent lymphocytes: r = 0.10, p = 0.31; percent neutrophils: r = −0.05, p = 0.60; percent eosinophils: r = 0.04, p = 0.69). The correlation with total cells was robust in that removal of the four greatest outliers had little effect on the correlation. However, visual inspection of the age versus total cells/ml (figure 7) suggests that the effect of age is not pronounced. Moreover, using r^2 as an estimate, age accounted for about 9.6% of the total variation.

Serial lavages of the same subjects demonstrated some variability over time, but the differences between the first and second lavages were normally distributed about the origin (figure 8). The mean difference was close to zero in all cases, and there was clustering about the mean, with rare outliers. There was a fair correlation between the cellularity of the first and second lavage of each subject (r = 0.67, p < 0.001) (figure 9), but there was no correlation between the cell composition of the first and second lavages (table 2 and figure 10).

The major difference between smoking and nonsmoking volunteers was that smokers had significantly more cells/ml return than did nonsmokers. (Smokers: median = 40.0, mean = 49.0 × 10^4 cells/ml; nonsmokers: median = 11.0, mean = 12.7 × 10^4; p = 0.001) (table 3). Smokers also had a higher percentage of macrophages and a lower percentage of lymphocytes (table 3).

Discussion

In order to utilize BAL as a clinical and research tool, it is necessary to define the distribution of cell counts in nondiseased, asymptomatic subjects. Our study indicates that the distribution of BAL cell counts in asymptomatic, nonsmoking volunteers is not normally distributed. Moreover, both the different methods used to express these results (i.e., percentage of cells, cells per 100 ml of infused BAL fluid, and cells per ml of returned BAL fluid) and the log transformation of the data do not appear to substantially affect these distributions.

The mean values observed in this study are in close agreement with the findings from other smaller studies (see table 4). As expected, macrophages were the most numerous type of cell present (93.3 ± 5.8%), and lymphocytes were the next most frequent cells present in BAL fluid (6.1 ± 5.6%). Although neither neutrophils nor eosinophils were seen in the majority of the lavages, 40% of the nondiseased, asymptomatic subjects had neutrophils and 13% had eosinophils. The mean percentages were 0.55 and 0.15%, respectively. The major difference between smokers and nonsmokers was a higher number of total cells in the smokers than in the nonsmokers (49.0 ×

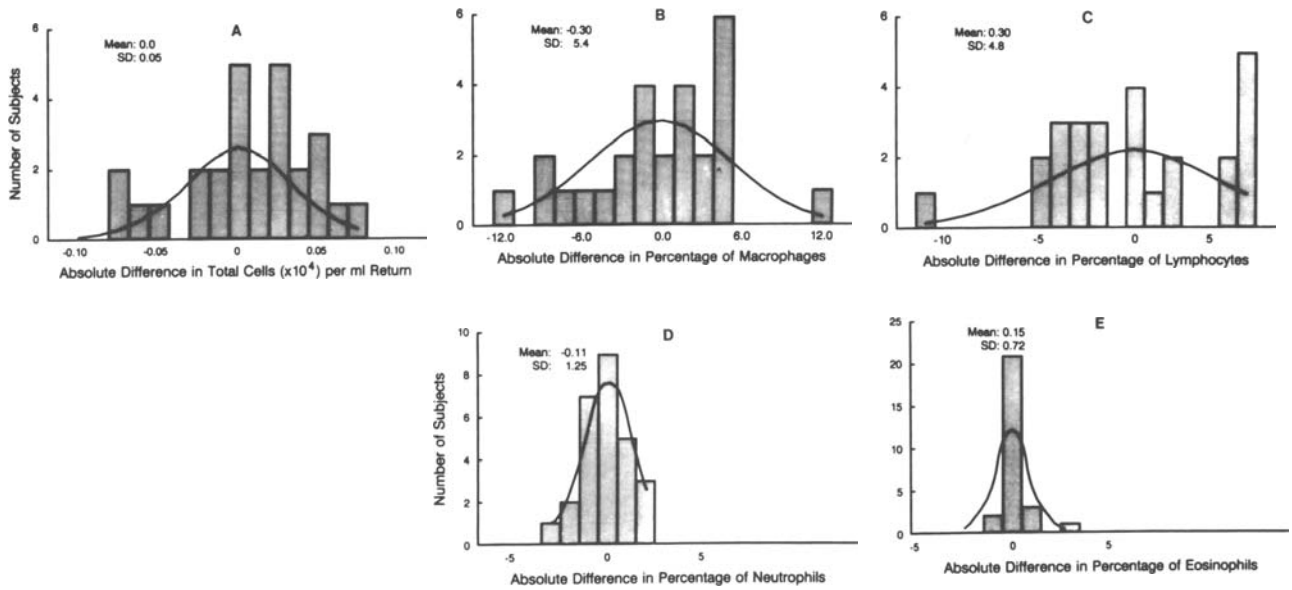

Fig. 8. Differences between the first and second BAL in 27 subjects who underwent serial lavages. Panel A shows a histogram of the differences in total cells per ml return. Panels B through E depict the differences in the percentages of the subtypes of cells. The normal curve is superimposed on each of the histograms.

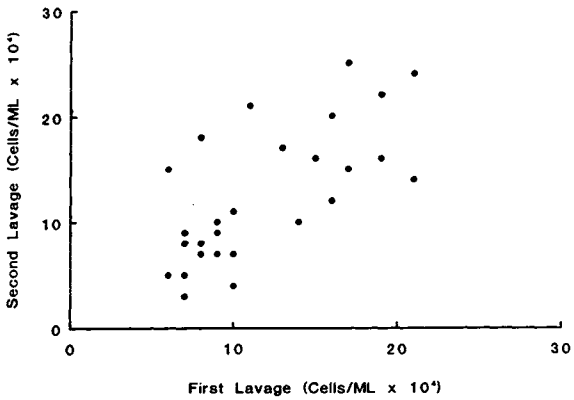

Fig. 9. Comparison of the first and second serial lavage total cellularity. There is good correlation ($r = 0.67$; $p < 0.001$) between the cellularity.

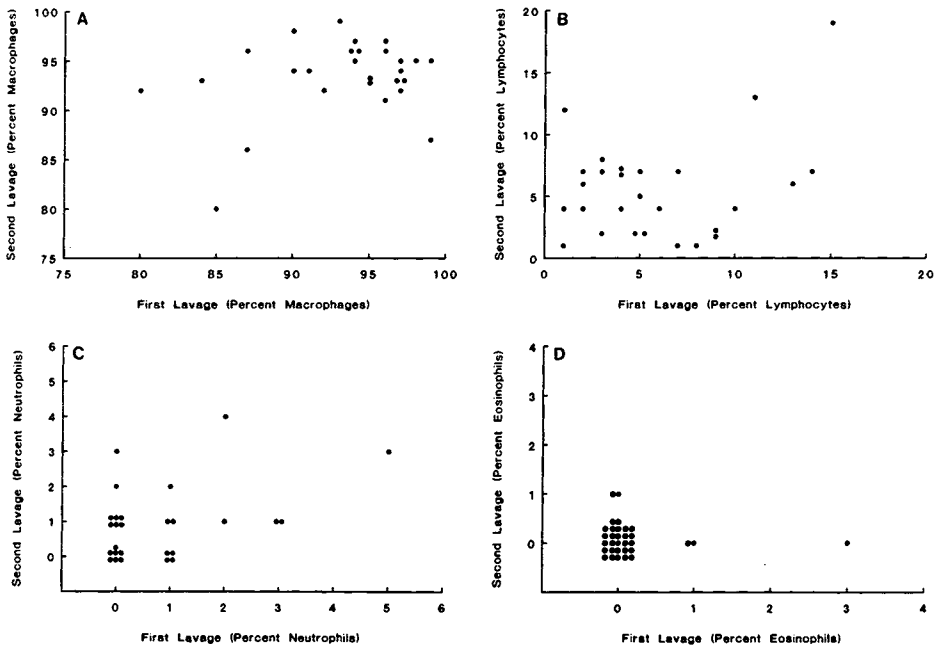

Fig. 10. Comparison of the first and second lavage cell percentages. In Panel A, percent macrophages are plotted for the first and second lavages. Panel B depicts percent lymphocytes. Panel C shows percent neutrophils. Panel D portrays percent eosinophils. There is no correlation between the first and second lavages for any of the cell percentages.

TABLE 3

COMPARISON OF BAL CELL COUNTS IN SMOKERS VERSUS NONSMOKERS*

	Nonsmokers ($n = 111$)	Smokers ($n = 19$)	p Value†
Cells/ml x 10^4	12.7 ± 9.1 (11.0)	49.0 ± 39.6 (40.0)	< 0.001
Macrophages, %	93.2 ± 5.8 (95.0)	98.6 ± 2.6 (98.0)	< 0.001
Lymphocytes, %	6.1 ± 5.6 (5.0)	2.16 ± 2.5 (1.0)	< 0.001
Neutrophils, %	0.5 ± 0.8 (0.0)	0.8 ± 0.9 (1.0)	0.21
Eosinophils, %	0.1 ± 0.4 (0.0)	0.2 ± 0.5 (0.0)	0.59

* Data expressed as mean ± SD with median shown in parentheses.
† p values calculated by Mann-Whitney U test.

10^4 versus 12.7 × 10^4). Although all cell types were increased in smokers, there was a disproportionate increase in the percentage of macrophages.

To use the BAL cell counts in population studies, it is necessary to know how the values are distributed around the mean. Statistical tests of the difference between two means, and tests of the likelihood that a particular value comes from the normal population, are dependent on assumptions about the distribution of the data. Student's *t* tests and analysis of variance, for example, assume that the data conform to a normal (bell-shaped) distribution. Our data indicate that BAL cell counts in nondiseased, asymptomatic subjects are not normally distributed. Analysis of the data showed that there is considerable clumping of the values. That is, there were more values close to the mean than expected and more values in the tails (the extremes) of the distribution than expected. These findings indicate that nonparametric statistical tests such as the Mann-Whitney U test should be applied to studies examining BAL cellularity.

Our findings are consistent with those of Laviolette (15) who reported that the percentage of lymphocytes in the lavage fluid of nondiseased, asymptomatic study subjects was not normally distributed. When he excluded the 12% of the subjects who made up the tail of the lymphocyte distribution (greater than 20% lymphocytes), the remaining data conformed to a normal distribution. This investigator concluded that, although his study subjects were clinically nondiseased, lymphocyte counts higher than 14% should be considered abnormal. Similarly, this is one possible explanation for counts comprising the long tails seen in our distributions. That is, the subjects with unusually high counts could have a subclinical illness at the time of the lavage. For example, one of our nondiseased, asymptomatic volunteers who had more than 15% lymphocytes on several occasions eventually developed an idiopathic Bell's palsy. It is possible that the Bell's palsy was a manifestation of the same immunologic process that caused the high lymphocyte counts. Another subject with an increased BAL lymphocyte count developed a viral upper respiratory infection three days after the lavage. At the time of the lavage, the subject was completely asymptomatic, clearly fitting the definition of nondiseased. On the other hand, other subjects with abnormal counts have been followed for

TABLE 4
COMPARISON OF BAL CELL COUNTS FROM DIFFERENT STUDIES OF NONSMOKING VOLUNTEERS

References	(n)	Age Range (yr)	Cell Return (× 10⁴/ml)	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)
Present study	111	20–48	12.7	93.2	6.1	0.54	0.14
Ettensohn, 1988 (11)	78	20–30	9.37	95.1	3.9	0.7	0.17
Baughman, 1986 (12)	16	16–68	N/A	92.2	6.6	1.3	–
Pingleton, 1983 (13)	10	24–35	20.6	89.9	7.7	1.7	0.6
Velluti, 1984 (14)	8	N/A	16.3	88.1	10.6	0.9	0.4

more than 5 yr without the development of clinical illness. Whatever the explanations of these data, we believe that it is inappropriate to use the results of the dependent variable (BAL cell count) being studied to modify the independent variable (clinical condition). That is, in this type of investigation, the lavage counts should not be used to reclassify nondiseased subjects as abnormal. A true random sampling of a clinically normal population, and even populations with lung disease, will include some individual subjects with subclinical illness such as viral illnesses. Exclusion of these subjects from the nondiseased population but not from the diseased population would result in a biased comparison. For most population studies, a nondiseased, asymptomatic subject is defined as a volunteer who is considered free of disease by predefined criteria. These criteria do not always exclude the possibility that the subject may be exposed to factors that could influence the variable being studied.

Our serial lavage data show a lack of correlation of cell composition between the first and second lavages. This suggests that the variability within individual subjects is similar to that between subjects. If the variability in our population were a result of different subgroups, with and without underlying disease, there would be significantly less variability within subjects than between subjects. The variability between subjects would be the sum of the within-subject variability plus the between-group variability. This is strong evidence that the variability observed in our population is an accurate estimate of the expected variability in a uniform population of nondiseased normal subjects.

Another important feature of this study and most other BAL studies is the age of the nondiseased, asymptomatic volunteers (11–14). These populations are usually characterized by young nonsmokers whose age may differ from the population with which it is compared. Although we found minimal effects of age in our study, further study is needed to better define the effects of aging on BAL cellularity. Differences in age, however, cannot explain the variability seen in this study.

There are other explanations for why the values may not follow a normal distribution. Normal distributions result from independent random sampling of continuous variables. Lack of independence, lack of randomness, or having a discrete rather than a continuous variable can all result in deviance from a normal distribution (10). The cell percentages actually represent discrete values based on counting a fixed number of cells. These data, therefore, would be expected to follow a Poisson distribution rather than a normal distribution. In fact, using the G test, there was excellent fit of the percentage of neutrophils and the percentage of eosinophils to the Poisson distribution. The goodness of fit to the Poisson distribution suggests that the eosinophil and neutrophil values are independent, random samples. However, the total cell counts are continuous variables and were not found to be normally distributed. Therefore, the deviation from a normal distribution in these cases results from either lack of independence or lack of randomness.

Lack of independence of the data could explain the observed clumping of the data. The milieu of the lung is thought to regulate the numbers and types of inflammatory and immune cells that are present. Inflammatory cells in the lung can effect the recruitment of additional cells. In various diseases, there is ample evidence that this type of regulation may occur (16, 17). This type of lack of independence would result in clumping of the data towards the mean and the tail, as we observed.

Regardless of its cause, lack of normality has important implications for statistical hypothesis testing. The parametric statistical tests such as Student's *t* test and analysis of variance, both assume a normal distribution (10). They are, therefore, inappropriate tests for analysis of these data, and their use could result in unexpectedly high type I error. Our findings indicate that it is more appropriate to use nonparametric tests, which do not require assumptions about the type of distribution.

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