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Research Article

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Pinocytosis by Human Alveolar Macrophages

COMPARISON OF SMOKERS AND NONSMOKERS

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Abstract Alveolar macrophages were obtained from human volunteers, smokers and nonsmokers, by bronchial lavage through a fiberoptic bronchoscope. Cells were incubated in a chemically defined medium containing [^{14}C]sucrose (0.36 mM) and varying concentrations of rabbit serum. Pinocytosis was assessed by the cellular uptake of isotope over 30, 75, and 120-min periods. Pinocytic activity of smokers' cells was dependent on serum concentration but always less than the activity of nonsmokers' cells. The degree of pinocytosis by nonsmokers' cells was independent of serum concentration. It is concluded that the decreased level of pinocytic activity in smokers' alveolar macrophages as indicated by the uptake of sucrose in the presence of rabbit serum may represent a form of reticuloendothelial blockade.

INTRODUCTION

Alveolar macrophages are of critical importance in defense of the lungs (1). Central to the defense function of these cells is their ability to take up foreign materials by endocytosis—either soluble substances or submicroscopic particles (pinocytosis), or microscopic particles or organisms (phagocytosis) (2).

Morphologic changes in alveolar macrophages from smokers, especially the presence of foreign inclusion material (3–6), suggest the theoretical possibility of some form of blockade of endocytic function of these cells. Studies with macrophages derived from the lungs of experimental animals do, in fact, reveal functional impairment of the cells by cigarette smoke or its constituents (7–9). To date, however, experiments with alveolar macrophages from human smokers have shown either no change or equivocal changes in the endocytic function of the cells (3–5, 10, 11). In the present study, we report data comparing pinocytosis in alveolar macrophages from human smokers and nonsmokers.

METHODS

Harvesting of alveolar macrophages. Subjects were between 21 and 39 yr of age of either sex, without significant symptoms or signs of respiratory disease, who had normal chest roentgenograms and ventilatory tests. Those in the smoker group had smoked at least one package of cigarettes a day for 5 yr. Anesthesia of the nasopharynx was accomplished with lidocaine. No other medication was administered.

An Olympus fiberoptic bronchoscope (12) was passed transnasally and positioned in one of the lower lobe segmental bronchi. The lung was irrigated with five 50-ml portions of warm physiologic saline (13) and lavage fluid withdrawn by gentle suction using a 50-ml syringe. Approximately 50% of the lavage fluid was recovered.

The recovered lavage fluid was strained through gauze and centrifuged at 270 *g* for 20 min, and the recovered cells were washed twice and finally resuspended in medium 199 (with Hanks Salts). Cell counts were done with a hemocytometer and cell viability was estimated by the determination of trypan blue due exclusion. Cell viability determinations were done before and after incubation of cells in all experiments described. None of the procedures resulted in a significant loss (> 10%) of viability.

For microscopic examination, a 1-ml aliquot of suspension of washed cells was added to a Leighton tube containing a flying coverslip. The medium was removed after 1 h and the attached cells were fixed in 1.25% glutaraldehyde at 4°C for 10 min. After fixation, the coverslips were mounted on glass slides and examined by utilizing phase-contrast optics.

Isopycnic density gradient centrifugation. In order to verify that human alveolar macrophages incorporate sucrose into pinolysosomes, subcellular fractions were prepared by isopycnic density gradient centrifugation. Approximately 15–30 × 10^6 cells were suspended in 25 ml of medium consisting of medium 199, 10% rabbit serum,[1] and 0.36 mM

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[1] To provide comparative data for future studies utilizing rabbit-derived antisera, rabbit serum, as opposed to autologous or homologous sera, was utilized throughout these studies. Preliminary studies were done comparing the effects of 10% rabbit serum, 10% homologous AB serum, and 10% autologous serum on sucrose uptake over a 75-min period. No differences in uptake were observed.

[^{14}C]sucrose (20 μCi/flask) in a spinner flask. The cells were incubated at 37°C for 2 h in an atmosphere of 5% CO_2 in air. Cells were recovered by centrifugation, washed three times, and finally suspended in 2.5 ml of 0.9 M sucrose solution. This was then homogenized in a glass homogenizer with a motor-driven Teflon pestle. The homogenate was centrifuged at 100 *g* for 2 min and the supernatant centrifuged at 27,000 *g* for 20 min. The final pellet was resuspended in 0.1 ml of 0.9 M sucrose solution containing 2% dextran (average mol wt 70,000) and layered above a linear sucrose: water gradient, 0.9–2.0 M, containing 2% dextran. This was then centrifuged at 204,000 *g* for 6 h in a Beckman model L2-65 ultracentrifuge with a SW 50 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). 16 fractions of 18 drops each were collected from the bottom of the tube. Radioactivity and acid phosphatase activity were measured in each fraction.

Determination of pinocytic activity. Aliquots of the suspension of washed cells were distributed among sterile plastic test tubes (Falcon Plastics, Div. of B-D Laboratories, Inc., Los Angeles, Calif.) so that each tube contained approximately 0.5–3.0 × 10^6 cells. The amount of cells in each tube depended upon the number in the original harvest. Media were removed by centrifugation and the cells resuspended in medium 199 containing 0.36 mM [^{14}C]-sucrose (3.3 μCi/tube) and the appropriate amount of rabbit serum. The suspensions were then saturated with 5% CO_2 in air and the tubes sealed and incubated in a shaker water bath at 37°C for 30, 75, or 120 min. At the end of the incubation period, the suspensions were chilled, washed twice with Hanks' balanced salt solution and the cells resuspended in water. Fractions were removed for protein and radioactivity determinations.

Analytical methods. Radioactivity was determined in a Nuclear-Chicago Mark II liquid scintillation spectrometer, utilizing a fluor solution containing toluene, 2,5-diphenyloxazole, 1,4-bis[2-(5-phenyloxazolyl)]benzene, and Triton X-100 (Packard Instrument Co., Inc., Downers Grove, Ill.). Samples containing undissolved protein were first digested in NCS Liquid Solubilizer (Nuclear-Chicago Corp., Des Plaines, Ill.) for 30 min at 37°C before addition to the fluor solution. Acid phosphatase activity was determined by a previously described method (14). Proteins were determined by the Lowry method (15) with bovine serum albumin as standard; DNA was estimated by the method of Bolognesi, Langlois, Sverak, Bonar, and Beard (16), using calf thymus DNA as reference standard.

Statistical analysis. The results are presented as mean ±SE. Comparisons were made based on the determination of L^2 utilizing the $F_{0.95}$ statistic (17). Rejection of the null hypothesis was assumed at $P = 0.05$ or less.

Reagents and media. Hanks' balanced salt solution, medium 199, and rabbit serum were obtained from Microbiological Associates, Inc., Bethesda, Md. Rabbit serum was filtered through a 0.45-μm Millipore filter before use. Uniformly labelled [^{14}C]sucrose was supplied by New England Nuclear Corp., Boston, Mass. Dextran (average mol wt 70,000) was obtained from Pharmacia Fine Chemicals, New Market, N. J. Bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, Mo., and calf thymus DNA from Schwarz-Mann, Bethesda, Md.

RESULTS

The number of cells recovered in the lavage fluid from smokers ($n = 13$) was 6.3±1.1 × 10^5 per ml, and from nonsmokers ($n = 13$) was 2.6±0.6 × 10^5 per ml, a significant difference. A similar difference has been noted by previous authors (3–6, 11). There was no difference in cell differential between smokers and nonsmokers (approximate 85% macrophages). No difference in cell viability between the two groups was observed. As others have reported (e.g., 6) the cell pellets of smokers looked a dark brown compared to the buff color of nonsmokers' cell pellets; on microscopic examination smokers' cells were larger, more vacuolated and pleomorphic, and had many phase- and light-dense cytoplasmic inclusions.

TABLE I

Effect of Iodoacetate on Uptake of [^{14}C]Sucrose by Alveolar Macrophages

Time	Specific activity	
	Control	Iodoacetate (10^{-3} M)
min	*dpm/μg of protein*	
30	1.35	0.39
75	2.48	0.50
120	3.33	0.91

Approximately 3 × 10^6 cells were incubated in medium 199 and 10% rabbit serum and 0.36 mM [^{14}C]sucrose with or without 10^{-3} M sodium iodoacetate at 37°C for varying intervals of time. At the end of the incubation, the reactions were stopped by chilling, the cells lysed, and uptake of radioactivity into cells was determined.

In order to demonstrate the energy-dependence of the uptake of [^{14}C]sucrose by macrophages, studies were done in which iodoacetate was interposed in the test system. Table I shows the results of an experiment in which cells from a nonsmoker were incubated with or without sodium iodoacetate, 1 × 10^{-3} M. It can be seen that there was a 71–80% inhibition of uptake of [^{14}C]-sucrose in the presence of inhibitor, which suggests that the uptake is an energy-dependent process.

Further evidence for the pinocytic uptake of sucrose was seen in the results obtained from the isopycnic density gradient experiments described above. These data are represented in Fig. 1. The radioactivity, in part at least, distributed with fractions containing the lysosomal marker enzyme acid phosphatase, indicating the presence of sucrose in pinolysosomes. The figure shown is from nonsmokers. Similar results were obtained with cells from smokers.

Cells from smokers and nonsmokers were then incubated with 1, 10, or 30% rabbit serum and [^{14}C]sucrose for 30, 75, or 120 min as described above. The results of these experiments are shown in Fig. 2. It can be seen that for each time interval and serum concentration, cells from smokers had lower uptake of radioactivity. When the data were replotted by the method of least squares in a way to emphasize the response of the two groups at each time interval to increasing levels of serum, only the smokers' cells showed a significant increase in radioactivity with increasing amounts of serum.

These data rely on a determination of specific activity, i.e., disintegrations per minute per milligram of protein. The possibility was considered that these results might have reflected an increase in the amount of protein in smokers' cells. DNA/protein ratios were determined for the two groups of cells. The ratio was 0.38±0.07 for smokers' cells ($n = 4$) and 0.23±0.02 for nonsmokers' cells ($n = 4$). The direction of the change, which was not statistically significant, actually favored less protein in the smokers' cells.

DISCUSSION

It appears reasonably certain that sucrose as used in these studies is an indicator, not an inducer, of pinocytosis. That is, induction of pinocytosis by serum or residual membrane factors (*vide infra*) results in the incorporation of sucrose into the pinosome as part of the entrapped extracellular fluid. Since sucrose is only slightly, if at all, susceptible to the hydrolytic action of lysosomal enzymes, it is retained within the cell for long enough periods to allow its use as a marker of pinocytosis. Cohn and Ehrenreich (18) have shown sucrose uptake by mouse peritoneal macrophages to require serum factors. Sucrose has also been used as an indicator of pinocytosis in a number of other cells, including those of rat (19) and human (20) liver, rat kidney (21), Chinese hamster fibroblasts (22), and rabbit alveolar macrophages (23). In the present studies, the uptake of sucrose by human alveolar macrophages was time and energy dependent, and the sucrose found in subcellular fractions with the approximate density of acid-phosphatase-containing organelles. In addition, when cells were washed before lysis and measurement of radioactivity, the final wash was essentially free of radioactivity. It appears, therefore, that sucrose is also an indicator of pinocytosis in human alveolar macrophages. Interestingly, Robin et al. (24) have used sucrose to measure *extracellular* water space in experiments with rabbit alveolar macrophages. The utilization of sucrose as an indicator of pinocytosis as well as of extracellular water is not the contradiction it appears to be.

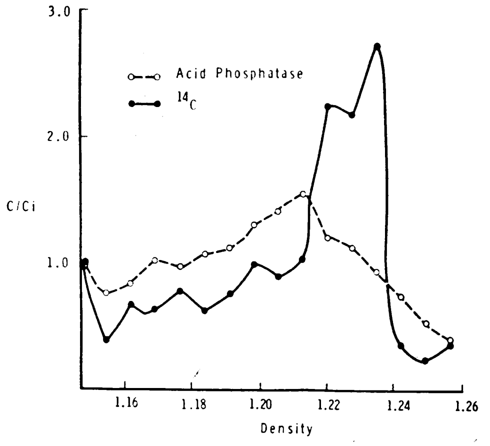

FIGURE 1 Distribution of sedimentable acid phosphatase and radioactivity from normal human alveolar macrophages incubated for 2 h in medium 199 containing 10% rabbit serum and 0.36 mM [^{14}C]sucrose. The relative concentration (ordinate) is the ratio of observed activity (C) to that which would have been found if the activity had been homogenously distributed throughout the whole gradient (Ci). Curves represent the means of three such experiments. Continuous sucrose: water gradient; 204,000 *g* for 6 h.

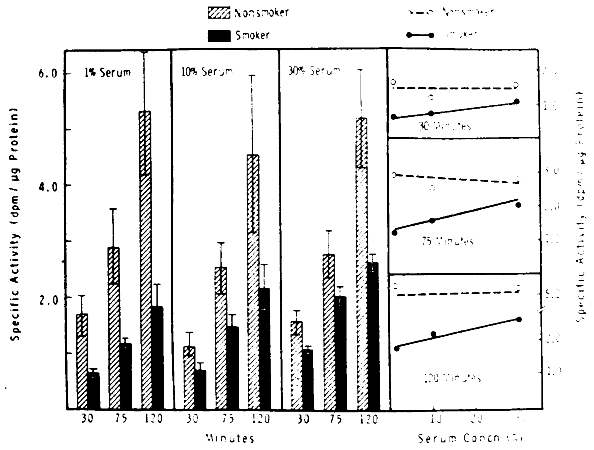

FIGURE 2 Pinocytic activity of smokers' and nonsmokers' alveolar macrophages in response to varying amounts of rabbit serum. *To the left:* Each bar represents the mean of the values obtained after a given time of incubation; the vertical lines indicate the standard error of the mean; $n = 4$ for smoker and nonsmoker groups on experiments performed with 1% and 30% serum; in the experiments with 10% serum, $n = 6$ for the nonsmoker group and 7 for the smoker group. *To the right:* The data are replotted by the method of least squares to show response at each time interval to varying concentrations of serum. A positive correlation exists for all the plots of data from smokers' cells.

The reduction in sucrose concentration of the medium due to pinocytosis by the cells was, at the most, only 0.0027% per mg of cell protein. Thus, when utilizing sucrose to determine extracellular water in the presence of cell concentrations and with the analytical methods described by Robin et al. (24), the error due to cellular uptake of sucrose by pinocytosis would be negligible, if at all determinable.

Two differences were observed in the pinocytic activity of smokers' alveolar macrophages when compared to that of cells from nonsmokers: (*a*) pinocytic activity appeared to be significantly diminished in smokers' macrophages; (*b*) the uptake of sucrose by smokers' macrophages was dependent upon serum concentration. The reasons for these differences can only be speculated upon. The most attractive explanations for these data would seem to be related to either reticuloendothelial blockade in smokers' macrophages, or loss of "endocytosis enhancing factor" in the lungs of smokers, or both.

Various alterations in morphology of smokers' alveolar macrophages have been noted in this study as well as by others previously. Pratt, Finley, Smith, and Ladman (3) described alveolar macrophages from smokers as having numerous inclusion bodies which impart a brown color to cell pellets. Harris, Swenson, and Johnson (5) identified some of the inclusion bodies as being fiber-like structures. Martin (6) remarked on the presence of fluorescent and autofluorescent intracellular material in smokers' macrophages. We were especially impressed with the extensive degree of vacuolation which occurred in smokers' alveolar macrophages. Such vacuolation occurred also in nonsmokers' cells to a lesser extent than seen with smokers but to a greater extent than that seen in rabbit alveolar macrophages.

It is likely that an exposure of the surface of the lung to an increased particle load stimulates production of free macrophages regardless of the nature (living or nonliving) of the particles (25). Such stimulation is probably related to an increase in the requirement for endocytic activity at the surface of the lung. It might be suggested, therefore, that smokers' alveolar macrophages have an increased level of endocytic activity *in situ* due to the particle burden of cigarette smoke. In mouse peritoneal macrophages, an increase in endocytic activity is accompanied by an increase in acid hydrolase production (26). In this connection, smokers' alveolar macrophages demonstrate an increased number of lysosomal organelles and increased acid hydrolase activity (5, 6). It is the increased particle load in smokers' cells where a basis for reticuloendothelial blockade may lie. Jeunet, Cain, and Good (27) have shown, for example, that after repeated phagocytosis of microorganisms by Kupffer cells of isolated perfused liver, reticuloendothelial blockade develops. They suggested that this was due to depletion of plasma opsonins or particular components of the surface membrane. The question is, what induced the pinocytosis observed in the studies described here—factors in the rabbit serum or membrane-bound humoral factors (cytophilic antibody ?) originating from the lung? Perhaps both. If the lung-derived factors attached to the membrane were more potent inducers of pinocytosis than factors in the rabbit serum, and if the smokers' cells were deficient in the lung-derived factors due to reticuloendothelial blockade, then this might explain the dependence of smokers' cells on serum concentration for pinocytosis.

The lung may also contain other factors which enhance endocytosis. Recently, Lentz and DiLuzio (9) reported that acellular lung wash from normal rats enhanced phagocytosis by alveolar macrophages, whereas wash obtained from rats exposed to smoke did not. Finley and Ladman (28) described a decrease in the lecithin content of lavage fluid from smokers compared to that of nonsmokers. Our studies of macrophage pinocytosis did not involve the presence of lung wash but this does not eliminate the possibility of residual effects of lung secretions on the cells.

The suggestion has been made that smokers suffer an increased susceptibility to infections (29–31). Previous studies of human alveolar macrophages have, to our knowledge, revealed no substantial depression of phagocytosis in the cells from smokers (5, 10). Reticuloendothelial blockade does demonstrate some specificity; in the studies by Jeunet et al. (27) blockade produced by *Salmonella typhosa* extended to *Brucella melitensis* but not to colloidal gold. It is also possible that acellular factors other than humoral (e.g., lung surfactant) may play a role in regulation of endocytosis by alveolar macrophages. Consequently, the interpretation of in vitro studies such as these with regard to the clinical situation must be approached with a great deal of caution.

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