ABSTRACT

Alveolar type II cells were isolated from five human lung specimens obtained during resection or lobectomy and enriched to 63-85% purity. Digestion with Sigma protease type XIV followed by centrifugal elutriation and Percoll density gradient centrifugation yielded 1.2 ± 0.4 x 10^6 cells/g lung in the type II cell fractions. The activities of some enzymes involved in the metabolism of xenobiotics were determined in these freshly isolated type II cells and compared with activities in alveolar macrophages and fractions of unseparated cells from the same tissue samples. Reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase activity was similar in the three cell fractions from all five patients (18-29 nmol/mg protein/min). An antibody to rabbit cytochrome c reductase inhibited reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reduction as much as 70% in microsomal preparations of the isolated human pulmonary cells, although this same antibody barely reacted with microsomes of the human cells in a Western blot assay. Epoxide hydrolase activity was highest in the alveolar type II cells (1.08 ± 0.17 nmol/mg protein/min). This activity was 6 times higher than in the alveolar macrophage or unseparated cell fractions. 7-Ethoxycoumarin deethylation activity, a cytochrome P-450-dependent pathway, was low or undetectable in the three cell fractions. Trace amounts of 7-ethoxyresorufin O-deethylase activity (0.5-1.5 pmol/mg protein/min) were detected in microsomes of the isolated human cells, even though a polycyclic hydrocarbon-inducible cytochrome P-450 which metabolizes 7-ethoxyresorufin (form 6 in rabbits) was not detected immunochemically.

INTRODUCTION

An important component of chemical carcinogenesis is the metabolic activation of procarcinogens, a process often involving multiple steps. Because of the structural and functional diversity of the many pulmonary cell types, studies of xenobiotic metabolism in isolated pulmonary cells may help us to understand the relationships between metabolic activation of procarcinogens and carcinogenesis at specific sites in lung.

Results of recent studies with cells isolated from rabbit and rat lungs have demonstrated differential cellular localization of several enzymes involved in the biotransformation of xenobiotics (1-7). In both rat and rabbit the nonciliated bronchiolar epithelial (Clara) cell catalyzes the metabolism of many compounds. In the rabbit, alveolar type II cells also have the ability to metabolize compounds, such as 7-EC, benzo(a)pyrene, and benzo(a)pyrene 4,5-oxide (2, 3). In contrast, alveolar type II cells from rats exhibit only traces of 7-EC deethylation and benzo(a)pyrene hydroxylase activities, and epoxide hydroxylase is apparently not detected in these cells (7).

In general, the human lung possesses low levels of enzymes that metabolize xenobiotics (8, 9). Cytochrome P-450 has not been detected spectrally, and cytochrome P-450 monoxygenase activities in human lung microsomes are less than 3% of those in human liver microsomes (8). Human pulmonary epoxide hydrolase activity is about one-tenth that of liver (8). However, one or more of the many lung cell types might have high activities of some of these enzymes. Studies of these enzyme systems in individual cell types of human lung have been limited to alveolar macrophages and lymphocytes, which are easily isolated but are not epithelial and exhibit low levels of xenobiotic metabolism. Attempts to correlate pulmonary carcinogenesis or cancer risk with elevated metabolism of xenobiotics, such as benzo(a)pyrene, in a single human cell type such as alveolar macrophages or lymphocytes have been unsuccessful (10, 11). Individual populations of human pulmonary epithelial cells, the stem cells of most pulmonary carcinomas, are more difficult to isolate and have not been used for these types of studies. Carcinomas of type II pneumocytes do occur in humans (12, 13). Therefore, study of xenobiotic metabolism by these cells is relevant to pulmonary carcinogenesis.

A procedure for the isolation of human alveolar type II cells, purified by differential attachment of cells to tissue culture plates and subsequent culture of the cells for 1 day, has been described recently (14). Studies of hepatocytes suggest that cultured cells rapidly lose cytochrome P-450 and exhibit changes in metabolism of xenobiotics (15). Preliminary experiments in our laboratory using rabbit tracheal cells support these findings. To avoid this potential problem, we developed a method of cell isolation from surgically obtained human lung tissue which yields a large fraction of freshly isolated pulmonary cells containing a high percentage of alveolar type II cells. This procedure uses a protease for cell dispersal, and elutriation followed by Percoll density gradient centrifugation for cell separation. Several reactions important in xenobiotic metabolism have been assessed in this fraction, and the activities were compared with those found in isolated alveolar macrophages and in mixed pulmonary cells from five individuals.

MATERIALS AND METHODS

Chemicals. Protease type XIV (lot 45F-0545) was obtained from Sigma Chemical Company (St. Louis, MO). The HPBS and the cell isolation buffer have been described previously (4).

Lung Tissue. Excess lung tissue was obtained from five patients undergoing clinically indicated lobectomy or pneumonectomy. Clinical details, occupational exposures, and drugs administered within 2 weeks of surgery are shown in Table 1. The tissue used was remote from the area of primary pathology and no macroscopic tumors were detectable. However, all the lung tissue contained abundant carbon particles. After lung cell fractionation, these were localized almost entirely in the macrophage fraction. Little or no carbon was observed in the type II cell fractions by light or electron microscopy. The lung tissue was kept in minimal essential medium on ice until cell isolation was begun, within 2 h of resection. Permission to perform this study was obtained from local committees responsible for protection of the rights of human subjects.

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1. To whom requests for reprints should be addressed, at Laboratory of Pharmacology (MD D4-04), NIEHS/NIH, P. O. Box 12233, Research Triangle Park, NC 27709.

2. The abbreviations used are: 7-EC, 7-ethoxycoumarin; HPBS, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid-buffered balanced salt solution; cyt c reductase, NADPH-cytochrome c reductase; P-450 reductase, NADPH-cytochrome P-450 reductase; 7-ERF, 7-ethoxycoumarin.

3. Unpublished data.
subjects (at both University of North Carolina and National Institute of Environmental Health Sciences).

Cell Isolation. Wedge-shaped sections of lung tissue weighing 26 to 109 g were placed in a 12-cm²-polystyrene dish and kept on ice during perfusion and instillation of protease. The cut surfaces of the tissue section were examined to locate airways and blood vessels large enough to insert a perfusion cannula (a blunted 20-gauge stainless steel needle attached to a repeating syringe). Airways were instilled with HPBS at 4°C and allowed to drain in order to remove alveolar macrophages. Blood was removed from the lung section by perfusing the vasculature with HPBS until the perfusate recovered was colorless. These washings were discarded. Protease solution (1.0% Sigma protease type XIV in HPBS) was instilled into both the airways and the vasculature, and the ends of the vessels were tied off with No. 00 silk sutures to prevent leakage. The protease-filled tissue was then transferred to a beaker and incubated at 37°C with gentle shaking for 20 min. This incubation time maximized yield of viable, dispersed cells, as shown by pilot studies with rabbit and dog lungs. The digested tissue was chopped, degassed for 30 s, and filtered as described previously for rabbit lung tissue (4). The filtered cells were centrifuged (800 x g for 10 min), resuspended in 10 ml cell isolation buffer containing 0.5% DNase to 10 ml, and filtered as described previously for rabbit lung tissue (4). The filtered cells were centrifuged (800 x g for 10 min), resuspended in 10 ml cell isolation buffer containing 0.5% DNase to 10 ml, and filtered as described previously for rabbit lung tissue (4). The filtered cells were centrifuged (800 x g for 10 min), resuspended in 10 ml cell isolation buffer containing 0.5% DNase to 10 ml, and subjected to centrifugal elutriation. A sample of unseparated cells, referred to as cell digest, was reserved to compare with separated cell fractions.

The details of the centrifugal elutriation system have been published previously (3, 4, 16). For preparation of human type II cells, three elutriator fractions were collected. The first fraction (150 ml) collected at 2200 rpm and 13 ml/min contained primarily blood cells and debris and was discarded. The second fraction (100 ml at 2200 rpm and 21 ml/min) contained 20-60% type II cells and was saved for further enrichment. The third fraction (100 ml at 1200 rpm and 18 ml/min) contained primarily macrophages (60-80% with less than 10% type II cells) and was also saved.

Cells from the second elutriator fraction were layered on a 35% solution of Percoll in HPBS and centrifuged at 1000 x g for 15 min. The cell fraction obtained from the top of the Percoll solution was enriched in type II cells. Following isolation and characterization, the different cell fractions were frozen in liquid N₂ and stored at -70°C until used for assays.

Cell Counts and Identification. Cells were counted with the Electrozone/Celloscope (Particle Data, Inc., Elmhurst, IL). Viability of cells was estimated by trypan blue (0.04%) dye exclusion (17). Alveolar type II cell and macrophage identification and enumeration were made using the modified Papanicolaou stain without acid alcohol (18) or by using phosphine 3R fluorescent dye (19). Type II cells were identified by the presence of stained cytoplasmic granules while macrophages were identified by their large size, round shape, and lack of stained granules. Clara cells were identified by electron microscopy (20). For electron microscopy, pellets of alveolar type II cells were fixed and processed by the method of Williams (21). Thin sections of cell preparations were examined in a Phillips model 300 electron microscope.

Enzyme Assays. All samples were sonicated (5 s at 60 W output with a Sonifer-Cell Disruptor; Ultrasonics, Inc., Plainview, NY) prior to enzyme assay. Microsomal preparations of isolated human pulmonary cells were used in some assays as indicated. Microsomes were obtained by centrifugation of sonicated cells at 10,000 x g for 20 min followed by centrifugation of the 10,000 x g supernatant at 100,000 x g for 45 min.

7-EC deethylase activity was assayed by fluorometric measurement of umbelliferone production. A modification of the method of Ulrich and Weber (26) was used. Sonicated cells were incubated with 0.4 mM 7-EC in HPBS with 3 mM MgCl₂ and in the presence of 1 mM NADPH for 15 min at 37°C. The reaction was stopped with trichloroacetic acid, and the protein was removed by centrifugation. The supernatants were made basic with Tris-glycine buffer, pH 9.0, and the unmetabolized 7-EC was removed by one extraction with heptane. The fluorescence of the umbelliferone formed was measured in the aqueous phase at an excitation wavelength of 375 nm and an emission wavelength of 458 nm.

The O-deethylation of 7-ERF was assayed in microsomal preparations of isolated human pulmonary cells by the method of Burke and Mayer (27) as modified by Normal et al. (28). Since microsomes were not washed, 10 μM dicumarol was added to the incubations to inhibit quinone reductase (29). The limit of detection in our system was 0.3 pmol resorufin produced/mg protein/min.

Enzyme activities were normalized to cell protein content [Bradford technique (30)] to facilitate comparison with published data on enzyme activities of human lung microsomes (8) and of cells from other species (2, 3, 6). In separate calculations, epoxide hydrolase and cytochrome c reductase activities were expressed per cell number because of size differences between macrophages (0.17 mg protein/10⁶ cells) and alveolar type II cells (0.08 mg protein/10⁶ cells).

Western blot analysis for immunoochemical detection of proteins was performed on both sonicated cells and microsomal fractions by the technique of Domín et al. (31) with antibodies prepared in goat to rabbit P-450 reductase and cytochrome P-450 form 6. These antibodies were kindly supplied by Dr. Richard M. Philpot, National Institute of Environmental Health Sciences. Pulmonary microsomes from rabbit or rat and purified reductase or cytochrome P-450 form 6 were analyzed in the same Western blot assays as positive controls for immunoreactivity of the antibodies. These same antibodies also showed immuno-reactivity with microsomes from human placenta of smokers (32).

RESULTS

The yield of cells in the cell digest following proteolytic digestion was 9.1 x 10⁶ ± 2.5 x 10⁶ (SE)/g lung tissue, and this fraction contained 10–30% alveolar type II cells. Similar numbers of alveolar macrophages and some polymorphonuclear leucocytes were observed, but other cell types in the mixed cell fraction were not identified. The alveolar type II cell fractions

---

**Table 1 Patient characteristics and type II cell purity for each specimen**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr)</th>
<th>Race</th>
<th>Sex</th>
<th>Pack years*</th>
<th>Current</th>
<th>Drugs</th>
<th>Occupational exposures</th>
<th>Pathology</th>
<th>Type II cell purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48</td>
<td>B</td>
<td>M</td>
<td>26</td>
<td>Yes</td>
<td>INH</td>
<td>Bronchiectasis</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>W</td>
<td>M</td>
<td>60</td>
<td>No</td>
<td>Methylclathizide</td>
<td>Large cell cancer</td>
<td>85</td>
<td>Large cell cancer</td>
</tr>
<tr>
<td>3</td>
<td>75</td>
<td>W</td>
<td>M</td>
<td>40 (pipe only)</td>
<td>No</td>
<td></td>
<td>Intermittent sand blasting, coal dust for 8 yr</td>
<td>65</td>
<td>Squamous cell cancer</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>B</td>
<td>M</td>
<td>15</td>
<td>Yes</td>
<td></td>
<td></td>
<td>Large cell cancer</td>
<td>65</td>
</tr>
<tr>
<td>5</td>
<td>47</td>
<td>W</td>
<td>F</td>
<td>20</td>
<td>Yes</td>
<td>Insulin</td>
<td></td>
<td>Squamous cell cancer</td>
<td>71</td>
</tr>
</tbody>
</table>

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* Number of years during which at least one pack of cigarettes was smoked/day.

B, black; W, white; INH, isoniazid.
isolated from these cells by the procedure described above contained $1.2 \times 10^6 \pm 0.4 \times 10^6$ cells/g of lung tissue. Of these cells, 74 ± 5% (range, 63–85%) were identified as type II cells (Table 1) and demonstrated a viability of 90% or greater by trypan blue dye exclusion. The major contaminants were alveolar macrophages and polymorphonuclear leukocytes. No Clara cells were identified in the type II cell preparations.

The type II cells were examined both by light microscopy (Fig. 1) and by transmission electron microscopy (Fig. 2). These isolated cells displayed the characteristic lamellar bodies and microvilli observed in human type II cells (14) and in type II cells isolated from other species (3, 6). The lamellar bodies from type II cells of human have concentric lamellae (Ref. 14; Fig. 2), whereas those of rats and rabbits contain cross-barred lamellae (3, 6).

The three different cell fractions from all five patients exhibited similar cyt c reductase activities (Table 2). In contrast, the epoxide hydrolase activities indicated a large and consistent difference between the cell types of human lung (Table 2). Epoxide hydrolase was detected in all the cell fractions, although the type II cells had about 6 times as much activity as the alveolar macrophages on a per mg protein basis. This activity varied only 2-fold in the type II cell fractions (0.7–1.4 nmol/mg protein/min) from the five patients.

Cytochrome P-450-dependent 7-EC deethylation reflects the activity of several cytochrome P-450 isozymes. 7-EC deethylase activities were low or undetectable in the cell fractions from the different patients. Only the mixed cell fraction from one individual had significant activity (169 pmol/mg protein/min). In all other fractions, the activity was less than 30 pmol/mg protein/min. In an assay to ensure that low 7-EC deethylase activity was not due to further metabolism of the product umbelliferone, no disappearance of umbelliferone (0.25 μM) was detected ($n = 5$ different cell preparations).

Western blot analysis was performed on the individual cell fractions and on microsomal preparations (for greater sensitivity) of the pooled type II cell or macrophage fractions in an attempt to detect immunochemically P-450 reductase or a polycyclic hydrocarbon-inducible isozyme of cytochrome P-450 (homologous to isozyme 6 in rabbit lung) in the isolated human cells. In rabbit lung this cytochrome P-450 isozyme is responsible for metabolism of both benzo(a)pyrene and 7-ERF (33). Antibodies made in goats to rabbit pulmonary P-450 reductase or hepatic cytochrome P-450 form 6, both of which react with human placental microsomes (32), were used for the analyses. Under conditions which were optimal for detection of the rabbit pulmonary proteins, neither antibodies to rabbit P-450 reductase nor to cytochrome P-450 form 6 formed identifiable bands with any of the human lung cell or microsomal preparations. (Maximum sensitivity of the assay detects 0.1 pmol/mg protein.)

Since a homologue of cytochrome P-450 form 6 was not observed immunochemically in the Western blot analyses, assays for 7-ERF deethylation were carried out with microsomal fractions of isolated human cells. Trace amounts of 7-ERF deethylase activity (0.5–1.5 pmol/mg protein/min) were detected in the microsomal preparations of macrophages, type II cells, and unseparated cells when 10 μM dicumarol was present in the incubations to inhibit quinone reductase activity.

Cyt c reductase activities in the human pulmonary cells (Table 2) were similar to reductase activities measured in rabbit pulmonary cells (3), but P-450 reductase (activity measured by
Fig. 2. Electron micrograph of isolated human alveolar type II cells showing characteristic lamellar bodies. × 15,000. Bar, 1 μm.

Table 2  NADPH-cytochrome c reductase and epoxide hydrolase activities in human pulmonary cells

<table>
<thead>
<tr>
<th></th>
<th>Cytochrome c reductasea (nmol cytochrome c reduced/mg protein/min)</th>
<th>Epoxide hydrolaseb (nmol benzo(a)pyrene 4,5-diol formed/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type II cells</td>
<td>22 ± 3 (1.7)b</td>
<td>1.08 ± 0.17 (0.082)</td>
</tr>
<tr>
<td>Macrophages</td>
<td>29 ± 7 (4.8)</td>
<td>0.18 ± 0.05 (0.028)</td>
</tr>
<tr>
<td>Cell digest</td>
<td>18 ± 2 (1.7)</td>
<td>0.23 ± 0.03 (0.022)</td>
</tr>
</tbody>
</table>

a Mean ± SE for 4–5 individual samples.
b Numbers in parentheses, nmol metabolite formed/10⁶ cells/min.

cytochrome c reduction) was not detected by the Western blot technique in microsomal fractions of the human cells. Therefore, we assayed cyt c reductase in the microsomes from human lung in the presence of the anti-rabbit P-450 reductase antibody (Fig. 3). Inhibition of the reductase enzyme in the human lung preparations was observed, but at a much higher concentration of the antibody than was required to inhibit the rabbit pulmonary enzyme.

Because inhibition of the human cytochrome c reductase activity by the anti-reductase antibody required a high antibody concentration, the Western blot analysis for detection of both the reductase and cytochrome P-450 form 6 was repeated with microsomes of the human cells and higher antibody concentrations (5 times greater than in the first experiments). With the anti-reductase antibody, faint bands were present in the expected molecular weight range with microsomes of both the macrophages and type II cells (data not shown). However, because of the higher level of nonspecific binding due to the increased concentration of antibody, these levels of staining were judged to be at best at the limit of detection and not quantifiable. With the cytochrome P-450 form 6 antibody, the faint bands did not stand out above the high level of background staining, and therefore detection under these conditions was not possible.

DISCUSSION

We have described a procedure for the isolation of human alveolar type II cells in sufficient number and purity to characterize several parameters of xenobiotic metabolism in the

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freshly isolated cells from small specimens (26–109 g) of excised lung. An important finding was the high epoxide hydrolase activity in type II cells relative to the other isolated pulmonary cells, indicating that human pulmonary cell types differ in their ability to metabolize chemicals.

The alveolar type II cell is a major pulmonary cell type representing about 16% of the cells of human lung (34). Recently, Robinson et al. (14) described a technique for isolation of human alveolar type II cells in which the tissue was digested with elastase. In our hands, elastase (0.05%) did not release as many human type II cells as did Sigma protease XIV (data not shown). Also, we were concerned that the isolation of cells from human lung, which contains little cytochrome P-450, be carried out as quickly as possible without a step involving differential attachment and culture of the cells. Therefore, in our procedure the cells were separated first using an elutriator centrifuge followed by Percoll density gradient centrifugation, a technique requiring a relatively short time (2–3 h).

For several years, our laboratory has been interested in isolation of nonciliated bronchiolar epithelial (Clara) cells from lung since in some species, they exhibit high rates of xenobiotic metabolism (2, 6). However, it was not surprising to us that few Clara cells were isolated in any of the cell fractions from the human lung tissue. Protease XIV does not yield many Clara cells from either rabbit or dog lung,3 and may selectively remove type II cells from human lung. Also, there may be a reduction in the number of Clara cells present in the bronchioles in response to smoking (35).

Epoxide hydrolase activity has been measured in microsomal preparations from human lung (8, 36). This very stable (as compared with cytochrome P-450) microsomal enzyme is important in the hydration of reactive epoxides. Epoxide hydrolase can play a dual role of activation and deactivation of reactive intermediates of polycyclic hydrocarbons depending on substrate concentrations and cytochrome P-450 isoforms present (37). Jones et al. (7) showed that type II cells from both untreated and β-naphthoflavone-treated rats exhibited no detectable epoxide hydrolase activity. However, our results suggest that human type II cells, unlike type II cells of rats, may function in metabolizing epoxides.

Cyt c reductase activity was similar in all the pulmonary cell fractions, approximately 20–30 nmol cyt c reduced/mg protein/min. In comparison, activity measured in sonicated type II cells of rabbits was 44 nmol/mg protein/min (3). The activity observed in the isolated human pulmonary cells was high enough to suggest that immunoreactivity might occur in protein blots with a goat antibody to rabbit P-450 reductase. In Western blot analysis to detect a homologue of the rabbit reductase in the microsomal preparations of isolated human pulmonary cells, the protein reacted minimally with a high concentration of the antibody. However, the same antibody was used to inhibit cytochrome c reduction as much as 70% in microsomal preparations of the human lung cells. These data provide evidence for the presence of cytochrome P-450 reductase in the human macrophages and type II cells.

7-EC deethylase activity was assayed in the isolated cells because it is a very sensitive measure of cytochrome P-450-dependent monoxygenase activity. Furthermore, several iso-enzymes of cytochrome P-450 metabolize this compound. Since most of the patients in this study were long term smokers, we also expected to detect a homologue of cytochrome P-450 form 6, which is present in placental microsomes of smokers (32). The lack of detection of this isozyme in the Western blot assay with human pulmonary cells of smokers could indicate a lack of immunological homology between the rabbit cytochrome protein and similar protein in human lung tissue. Indeed, traces of 7-ERF deethylase activity, which has been correlated with the presence of this isozyme in human placental tissue of smokers (32), were found in microsomal preparations of all three human pulmonary cell fractions. However, the low or undetectable 7-EC deethylase activities as well as the Western blot data with an antibody to cytochrome P-450 form 6 and the low 7-ERF deethylase activities measured in the isolated human pulmonary cell preparations are consistent with previous results showing little or no cytochrome P-450 or cytochrome P-450 monoxygenase activity in human lung microsomes (8).

The tissue obtained for these studies, although lacking grossly detectable tumors, came from cancerous lungs. Similarly, other studies on xenobiotic metabolism in human lung have been performed with "normal" tissue from diseased lungs (8, 9). In rat livers containing 4-dimethylaminoazobenzene-induced tumors, tissue adjacent to the tumors had normal monoxygenase activity whereas activity in the tumors was significantly decreased (38). This suggests that cells from normal lung tissue of patients with lung cancer may have the same enzyme activities as those from people without disease. Indeed, the cells from the subject with bronchiectasis but not cancer had enzyme activities comparable to the four patients with lung cancer. Further studies with human lung epithelial cells, such as alveolar type II or Clara cells, may reveal differences in metabolism between cells from the normal population and normal or tumor cells from lung cancer patients.

In this report, we have shown that type II cells can be isolated from pieces of excised human lung tissue in sufficient quantity to measure the activities of several enzymes in the freshly isolated cells of one individual. The epoxide hydrolase data demonstrated that marked differences in the activity of at least one enzyme involved in xenobiotic metabolism exist between different human lung cell types. These isolated cell preparations may be useful for the localization and study of endogenous substrate metabolism, such as biotransformation of arachidonic acid and prostaglandins, since many of these pathways do not involve cytochrome P-450.

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Xenobiotic Metabolism in Human Alveolar Type II Cells Isolated by Centrifugal Elutriation and Density Gradient Centrifugation

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