Isolation of Reed-Sternberg Cells From Lymph Nodes of Hodgkin’s Disease Patients

By Giammaria Sitar, Ercole Brusamolino, Carlo Bernasconi, and Edoardo Ascarì

This study describes a simple and relatively rapid method of purifying Reed-Sternberg (R-S) cells and their morphologic variants from the lymph nodes of patients affected by Hodgkin’s disease. Our initial studies defined the optimal procedure for a quantitative disaggregation of Hodgkin’s lymph nodes and the densities of R-S cells in several donors. These preliminary steps were helpful in the development of strategies for selectively concentrating R-S cells by density gradient centrifugation. We layered a single-cell suspension over Percoll of appropriate density, centrifuged these samples for 15 minutes, and collected a fraction enriched in R-S cells. Most of the R-S cells were distributed between densities of 1.060 and 1.072, with a peak at approximately 1.068 g/mL. R-S cells are denser than many mononuclear cells present in the lymph nodes of Hodgkin’s patients and lighter than reactive cells such as eosinophils, mast cells, and neutrophils. However, the ranges of densities of these cell types overlap, making purification of R-S cells by isopycnic centrifugation impossible. Nevertheless, when this enriched fraction is further processed by velocity sedimentation in order to take advantage of the larger size of R-S cells as compared with all other cells, a substantial purification is achieved. We used three different velocity-sedimentation chambers to find the optimal conditions for obtaining the highest purity with a high final yield. The cells isolated by this method are viable, appear to be morphologically normal, and have been further characterized biologically.

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MATERIALS AND METHODS

Cases. Fresh tissues were obtained from the operating room and processed immediately. Consecutive lymph node sections of untreated Hodgkin’s disease, as diagnosed in the Department of Pathology, University of Pavia, were studied before treatment. Seven patients had a histologic diagnosis of nodular sclerosis, one of mixed cellularity, and one of lymphocytic depletion pattern.

Disaggregation of lymph node into a single-cell suspension. The full procedure has been detailed previously. Briefly, lymph node material was dissected free of capsule and small vessels with a fine razor blade, washed thoroughly in Hank’s balanced Ca-Mg-free salt solution (HBSS) to remove blood and debris, and weighed and cut at 250-µm thickness with a Smith-Farquhar tissue slicer (Dupont Co, Newtown, CT). Slices were suspended in a dissociation chamber consisting of a leucite reactor with a water jacket. The reactor has three inlet ports on the bottom and a spillover port on the top. The flow rate through the dissociation chamber is controlled by a peristaltic pump set at 25 mL/h. The concentration of crude collagenase, hyaluronidase, and deoxyribonuclease-(DNase) (Boehringer, Mannheim, FRG) in HBSS was 2 mg/mL, 1 mg/mL, and 50 µg/mL, respectively. An upward fluid movement is obtained by the special design of the reactor’s bottom part and the use of a U-shaped magnetic stir bar (Bel-Art Products, Pequannock, NJ). As soon as cells are freed from the tissue slices, they are forced by the upward velocity component through a 150-μm polyester monofilament screen (placed across the top of the reactor) and into a delivery vessel containing Roswell Park Memorial Institute (RPMI)
1640 medium and 10% fetal calf serum (FCS) (Gibco, Breda, The Netherlands). Cells are then washed to minimize residence time in contact with the dissociating enzymes.

Collagenase was warmed to room temperature before use, and the amount of the clostripain inhibitor tosyl-L-lysyl-chloromethane (TLCM) (Calbiochem, La Jolla, CA) required to fully inhibit the clostripain activity in the crude collagenase preparation was added, since this enzyme has been shown to be most destructive to cells in the isolation procedures.\textsuperscript{41} Incubation was allowed to proceed for 20 minutes before the beginning of tissue digestion.

**Quantitation of R-S cells in suspension.** The quantitation of cells was performed at different steps in the purification procedure by optically enumerating nucleated cells in a hemocytometer (improved Neubauer chamber). R-S cells and other cells were counted at a magnification of 160× with phase-contrast optics. R-S cells were identified by their larger size, the presence of a large nucleus or two or more nuclei, and the presence of one or more prominent nucleoli. A differential count was made on cyt centrifuge preparations (cytology buckets, IEC) stained with May-Grünwald Giemsa (MGG). At least 200 cells were counted on each smear.\textsuperscript{42}

Cell viability was assessed by the trypan blue dye exclusion test. Cell diameter was measured in cyt centrifuge preparations stained with MGG with use of a light microscope with a graticule inserted into a 10× eyepiece (obj 100×). Fifty cells were measured, and mean size ± SD was calculated for each cell type.

Acid phosphatase and nonspecific esterase were studied in cyt centrifuge preparations stained according to recommended procedure.\textsuperscript{42}

**Separation of cells by isopyknic-gradient centrifugation.** Percoll stock solution (SS) (Pharmacia, Uppsala, Sweden) was prepared by mixing 90 mL of commercial Percoll with 8.96 mL of 10× HBSS without Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, 0.45 mL (1N) HCl, and 1 mL Hepes buffer (1 mol/L). The required density of Percoll was prepared according to the following formula:\textsuperscript{43} Percoll density (g/mL) = (\% Percoll SS × 0.001186) + 1.0041, where 1.0041 is the density of the physiologic media. The final density of the Percoll solutions was adjusted by measuring with a densitometer (K. Paar, AG, Graz, Austria). All solutions used for isopyknic centrifugations were adjusted to 300 mOsm with 4 mol/L NaCl or distilled water and to pH 7.3 with 1N HCl or 1N NaOH. The osmolality of the solutions was determined with the freezing-point depression technique. Continuous density gradients were prepared by centrifuging Percoll 65% SS in an ultracentrifuge (model L5-50B, Beckman) with a 23°-angle head rotor at 30,000 g for 15 minutes. Density marker beads (Pharmacia, Uppsala, Sweden) were run in parallel to check the density gradient. Cells were resuspended in HBSS containing 0.05% polyethylene oxide (PEO) (British Drug House, Poole, England) before being applied on the density gradient, as shown in Fig 1 and as described by Leif.\textsuperscript{44}

Discontinuous gradients were prepared by overlayering, with a peristaltic pump, 6 mL of Percoll solutions of decreasing densities (1.090, 1.080, 1.070, 1.060, and 1.050 g/mL). Cells were centrifuged at 2,000 g for 15 minutes at room temperature in a benchtop centrifuge. To avoid distribution disturbances, the rotor was slowly accelerated manually according to the time span suggested by Leif,\textsuperscript{45} and the run was stopped without braking. After centrifugation, cells were collected from the bottom of the tube with use of a long needle and a peristaltic pump. The cells were then washed in HBSS, counted, and examined under phase-contrast microscopy for the presence of R-S cells. R-S-enriched fractions were pooled and further processed by velocity sedimentation.

**Separation of cells by velocity sedimentation.** In pilot experiments we made use of three different chambers, which have been previously described in extenso.\textsuperscript{31,32,46} The sedimentation chamber used in the purification of human megakaryocytes\textsuperscript{39} proved superior to the other models, both in results and ease of handling; therefore, the separation procedure will be described with reference to this model. The separation chamber is filled from the bottom with a linear density gradient generated through a three-channel peristaltic pump (P-3, Pharmacia, Uppsala, Sweden). The gradient is underlaid with a dense liquid that is immiscible with water (Fluorinert, 3M Co, St Paul, MN), which brings the gradient up to the end of the capillary tube (Fig 1). Next, the cell sample is introduced into the upper chamber and the peristaltic pump is reversed, thus lowering the band of cells to the cylindrical part of the device. Before layering the cell suspension onto the density gradient, the cells are counted in a hemocytometer to ascertain that the cell concentration is well below the "streaming limit," which for this apparatus is 90 × 10\textsuperscript{6} cells. Cell counting is not performed with an automatic apparatus, to avoid having debris or small-sized particles interfere with the electronic counting. This procedure yields a very thin band (less than 1 mm) of an undisturbed cell suspension on the gradient. Cells are allowed to sediment at unity gravity for three hours.

**Immunophenotyping**  Cell surface antigens were detected by a standard indirect immunofluorescence (IF) assay. Monoclonal antibodies T6 (CD1), T11 (CD2), and OKM1 (CD11b) were obtained from Ortho Diagnostic (Raritan, NJ), B1 (CD20) from Coulter Immunology (Hialeah, FL), and anti-common lymphocytic leukemia antigen (CALLA) (CD10) from Becton Dickinson (Mountain View, CA). Immunolabeling of centrifuged slides with Ki-1 monoclonal antibody (Dakopatts, Copenhagen) was performed by a three stage immunoperoxidase method with use of a Dako monoclonal kit (Dakopatts, Copenhagen) according to the manufacturer's instructions. Additional studies included testing for surface and cytoplasmic immunoglobulins with use of fluorescence-labeled rabbit antihuman antiserum (Dakopatts, Copenhagen). Staining was performed for kappa and lambda light chains and for gamma Ig chains.

**Short-term culture.** After separation, cell fractions containing R-S cells were pooled and washed twice in medium. For short-term culture, cells were placed in RPMI-1640 containing 25 mmol/L Hepes buffer, 1% antibiotic antimitotic mixture, 2 mmol/L glutamine, and 10% FCS (all from Gibco) at a concentration of 2 × 10\textsuperscript{5} cells/mL. SS, stock solution; ly, lymphocytes.

![Fig 1. Isolation of R-S cells from lymph nodes of Hodgkin's disease patients by a two-step procedure. SS, stock solution; ly, lymphocytes.](image-url)
cells/mL and aliquoted into a multiwell tissue-culture plate (Costar, Cambridge, MA). The plates were incubated at 37°C in a humidified atmosphere of 5% CO₂, 95% air. After 24 hours in culture, cells were harvested by withdrawal of the well content with a plastic pipette, then washed and examined.

RESULTS

Cell yield after lymph node disaggregation. Dispersion of tissue samples with the enzyme combinations yielded an average of $2.5 \times 10^7$ (range, 1.8 to 4.5) cells per gram (wet weight) of tissue. Besides a predominant small lymphocyte population, we observed nucleolated lymphoblasts and a variable amount of medium-large lymphoid cells with strongly basophilic cytoplasm; eosinophils and monocytes were present in various amounts, mast cells were mainly present in the nodular sclerosis subtype, and large granular lymphocytes could also be found in very small percentages (<1%). It was outside the scope of this study to characterize all the cell types present in the disaggregated tissue samples and to analyze their density distribution profiles in our experimental system.

R-S cell concentration after isopyknic centrifugation. Isopyknic-gradient centrifugation resulted in a substantial enrichment of R-S cells (Table 1). The density distributions of R-S cells and several other cell types in a typical experiment are shown in Fig 2. Large activated lymphocytes, monocytes, lymphoblasts, and eosinophils are distributed in the lighter and denser parts of the gradient, respectively, whereas the intermediate part is largely made up of two diffuse bands that contain mostly R-S cells and small lymphocytes.

It must be taken into consideration that the absolute shape of the individual peaks is by no means precise, both because of the limited number of cells that are counted and because of the wide structural heterogeneity of R-S cells, which brings about a heterogeneous density distribution. Mean cell density, size, and sedimentation velocity in our experimental conditions are shown in Table 2. For preparative purposes, we discarded both the low-density (<1.060) and high-density (>1.072) cell fractions, as shown by density-marker beads run in parallel, and we collected the R-S enriched cell fraction in the intermediate region of the gradient (Fig 2).

R-S cell purification after velocity sedimentation. R-S cells were further purified by processing the intermediate cell fraction by velocity sedimentation. The best results were obtained with the same chamber used previously in the purification of human megakaryocytes. After three hours of sedimentation, most R-S cells were recovered in the fast-moving fractions, together with a few small lymphocytes bound to the R-S cells. Fig 3 shows the velocity-sedimentation distribution of the intermediate-density cell fraction in a typical experiment. The large distribution profile of R-S cells is due to the heterogeneity of tumor cells with respect to size (16 to 36 μm). In contrast, the mononuclear cell fraction always yielded a relatively narrow peak at the sedimentation velocity of 6 mm/h. A clear-cut separation between the two cell populations (ie, R-S cells and all other cells) is obtained with only minimal contamination due to the lymphocytes firmly bound to R-S cells. Figure 4 shows the final purity achieved after the two-step procedure. The recovery varied and in some cases approached 75%; some cells were lost during fraction collection because they stuck to the lower cone, as was experimentally verified with use of colored volume-marker beads (Coulter Electronics, Hialeah, FL), and others during the washing procedures. Purification was achieved in three hours, but cell concentration and counts on individual fractions extended the time to five hours.

Cytochemical and immunophenotypic analysis. Table 3 summarizes the cytochemical and immunophenotypic data for our purified cell fractions. Consistently positive staining was present for acid-phosphatase activity, whereas alpha naphthyl acetate esterase was positive in most but not all R-S cells and showed a diffuse brown coloration with a localized accumulation of brown granules. Many R-S cells and their variants stained positive for IgG in all cases, with clear staining of positive cells and minimal or absent background staining. Plasma cells colored for comparison always showed a more intense staining. Cytopreps stained for kappa and lambda chains showed that the majority of R-S cells stained for the kappa chains and fewer

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<th>Table 1. Quantitative Results of Concentrating R-S Cells From Hodgkin's Disease Lymph Nodes (Results of Seven Experiments)</th>
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Fig 2. Buoyant density distribution of R-S cells and other cell types obtained from a disaggregated lymph node. 150 x 10^6 cells were applied on a 30-ml density Percoll gradient. After centrifugation at 2,000 g, 1.5-ml fractions were collected and differential count performed on cytoperps. Gradient was monitored with density-marker beads. Ly, lymphocytes.
stained for the lambda light chains, especially in cases of nodular sclerosis subtype. A large proportion of the R-S cells and their variants were also positive when stained with Ki-1 monoclonal antibody in the two cases tested, but the intensity of staining varied. In no case was monoclonal expression of kappa or lambda chains demonstrated. Monoclonal T-cell antibodies and the antimonocyte antibody OKM1 were entirely negative, whereas in three cases of the nodular sclerosis subtype, positive staining with B1 antibody was present. A more comprehensive immunophenotypic analysis was not possible, since there was insufficient material to permit this.

Short-term culture. After 24 hours in culture, R-S cells appeared to be morphologically intact and were able to form rosettes after incubation with autologous lymphocytes, though the number of adherent lymphocytes was less than that found immediately after tissue disaggregation.

DISCUSSION

These studies were designed to develop a suitable method for purifying R-S cells from tumor material in sufficient quantity and of adequate purity for experimental work.

Many previous studies have shown that cells of a given volume may be heterogeneous in density and that cells of a given density may have a wide volume distribution. These considerations have suggested that successive density-based and sized-based separation procedures will frequently be fruitful.\(^5\) As Miller wrote, "The analogy with molecular separation, in which various physically based techniques, such as centrifugation and electrophoresis, are used both preparatively and analytically, is obvious." Pretlow et al\(^5\) and Wilson et al\(^5\) separated different kind of cells from Hodgkin's disease spleens and lymph nodes but were able to purify only the lymphocytes and not the R-S cells. Payne et al mention using a modification of the gradient-sedimenta-
sition method of Pretlow to concentrate R-S cells, but they do not describe details or results.56 Olsson has attempted to isolate the giant Hodgkin’s cell from biopsy specimens, but here again, no details of methodology or results are provided.4 Kadin et al7 concentrated R-S cells by a one-step procedure through velocity sedimentation at unity gravity, using Miller’s techniques.52

This lack of methodology is presumably due to one major technical problem: the disaggregation of Hodgkin’s lymph nodes into single-cell suspensions. These lymph nodes are often the site of extensive fibrosis; indeed, several investigators have underlined the difficulty of obtaining a large number of viable cells.12,17,31,55 We have overcome this problem previously and shown how to obtain a large number of viable cells suitable for cell fractionation by physical methods.

The strategy for obtaining the purification of R-S cells was based on several investigations of the cellular composition of normal and Hodgkin’s lymph nodes12,16,56 and on data that were already available on the physical properties (density and size) of some of these cells. These studies showed that lymphocytes from normal lymph nodes have a wide density-distribution profile and that two peaks are found in the 1.062 and 1.072 g/mL regions. They further confirmed previous observations that lymphocytes change their density markedly upon activation, moving toward the low-density region. De Pauw et al36 have studied the buoyant density-distribution profile of lymphocytes from Hodgkin’s disease patients and have found that relatively more lymphocytes have a low specific gravity in comparison with those of normal controls. Monocytes,39 NK cells,64 and lymphoblasts peak in the low-density region (<1.060 g/mL), whereas eosinophils,52 RBCs, and mast cells peak in the high-density region (>1.072 g/mL).

On the basis of these and our own preliminary results,46 we chose a very peculiar continuous-density gradient (S-shaped), in which the intermediate region is artificially expanded while both the low- and high-density regions are compressed (this being irrelevant for our purposes). Continuous gradient provided a better enrichment of R-S cells than did step gradients because the interactions between R-S cells and T lymphocytes are substantially reduced. Leif has gone so far as to state that “unless there is some overriding preparative reason and the system has been already well-studied with linear gradients, the results obtained with step gradients are not suitable for publication in reputable journals.”64 We think that the use of discontinuous gradients, even for preparative reasons, should be discouraged in these particular circumstances because of the strong tendency of R-S cells and lymphocytes to aggregate spontaneously.63 McGuire and Pretlow have reported that “more than 45.8% of the Hodgkin’s cells in the purified fractions from the gradients had attached lymphocytes.”64

Lepoivre and Lemaire52 have investigated the intercellular binding strength between cytolytic effector cells and their tumor target. Although the association of T lymphocytes around R-S cells is of unknown origin, with use of the disrupting conditions suggested by these investigators, the intercellular association of these rosettes appeared weakened, since most but not all the rosettes were disaggregated. We have not tried higher g fields, since the effects of differences in the compressibility of the cells and the medium would alter the distribution.44

The theory underlying cell sedimentation holds that the rate of sedimentation is largely a function of cell size.52 Therefore, it seemed logical that velocity sedimentation should be an appropriate method of purifying R-S cells. The isokinetic gradient of Pretlow, which he centrifuged at low g forces (97 g), has two major drawbacks that are serious impediments to the sedimentation of cells in a centrifuge tube. The first is the “wall effect,” which is due to radiation of the cells from the axis of rotation to the walls of the tube, where they adhere or slide down as a mass to the bottom of the tube. In ordinary tubes, cell loss may be as high as 60%; positioning the tube far from the axis of rotation reduces but does not eliminate this effect. The second drawback is the swirling movements of liquid upon acceleration and deceleration. Theoretical details have been discussed elsewhere, and technical improvements to overcome these drawbacks have been proposed.46

Separation of the intermediate-density fraction by velocity sedimentation was successful, regardless of which of the three sedimentation chambers was used. The purity of large R-S cells was excellent because of their much larger size as compared with all other cells present in the intermediate-density fraction. It is not impossible that some large reactive cells contaminated the isolated R-S cell fractions, since not all giant cells fulfill the classic morphologic criteria of R-S cells and their mononuclear variants. Interdigitating cells may occasionally resemble R-S cells, although the channel-like invaginations of the nuclear membrane and the numerous interdigitating fingerlike processes are typical hallmarks of these cells.65,66 Macrophage-histiocytes may also resemble tumor cells in Hodgkin’s disease, but large macrophage populations only occasionally attain the size of R-S cells.66,69 Haskill et al have separated mononuclear cells infiltrating

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Abbreviations: ANAE, alpha-naphthyl-acetate-esterase; CD, cluster designation described at the Third International Workshop on Human Leukocyte Differentiation Antigens (1986).47

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ovarian cancer from tumor cells by velocity sedimentation, and in only a few cases "were tumor cells similar in velocity sedimentation to the largest, most rapidly sedimenting inflammatory cells (<6/mm/h)." Nevertheless, a more comprehensive immunologic study will be necessary before any conclusions can be drawn from results obtained with use of these cell fractions.

The immunologic and cytochemical investigation of our purified cell fraction was limited by the amount of cellular material available and did not permit us to draw any conclusions regarding the nature of R-S cells.

Recently, molecular probes have been introduced to aid in the diagnosis and classification of lymphomas. Conflicting results have been obtained in preliminary studies on Hodgkin's disease. Possibly because of the very low number of neoplastic cells in unseparated cell suspensions. Shortly before this manuscript was submitted, two papers appeared concomitantly on molecular genetic analysis of Ig and T-cell receptor genes, and both drew attention to two major technical problems: that rearrangements in minor cell populations may remain undetected, since they are below the threshold levels of Southern blot analysis, and that the clonal cells detected by this approach may represent a monocular lymphocyte population contaminating the cell fraction. Furthermore, it was stated that "documentation of gene rearrangements of R-S cells will necessitate the elimination of extraneous cells by further cell purification." The procedure described in this paper may prove useful in this respect because it provides an almost homogeneous cell fraction.

This cellular material represents a favorable alternative to long-term established cell lines for studying the cell biology of R-S cells. It should be kept in mind that patients who have served as donors of successfully established in vitro cell lines have often undergone heavy treatment with radiotherapy and/or chemotherapy, which may conceivably have selected atypical malignant cell populations. Olsson and Behnke have emphasized that clonability of a cell culture is a prerequisite for defining a culture as a cell line. The necessary selection of one clone as the basis for the establishment and expansion of the line always carries the risk of establishing a cell line from an atypical clonal cell of the malignant population and, consequently, the possibility of obtaining a line with atypical phenotypic attributes. These conflicting results might be further explained by the possibility that the methods of culture used may have selectively enhanced the emergence of different cellular lines with different cell features, and/or by the possibility that established cell lines, by virtue of either genetic or epigenetic adaptation to tissue culture conditions, may no longer resemble their alleged counterparts in vivo.

In conclusion, we have described the first method of obtaining highly pure preparations of R-S cells from the lymph nodes of patients affected by Hodgkin's disease. We expect that this procedure will prove useful for a detailed investigation of the biochemical and functional properties of R-S cells.

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