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Authors
Lundak, RL
Runge, PE
Granger, GA

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SEPARATION OF FUNCTIONAL SUBPOPULATIONS OF MURINE AND HUMAN LYMPHOID CELLS ON COLLOIDAL SILICA DENSITY GRADIENTS. I. PREPARATION OF THE LUDOX AM® GRADIENT MATERIAL AND CHARACTERIZATION OF SEPARATION CAPACITIES

R.L. LUNDAK *, P.E. RUNGE ** and G.A. GRANGER **

* Division of Biomedical Sciences, University of California, Riverside, CA 92521, and
** Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 90266, U.S.A.

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This report describes a unique modification of an isopycnic density gradient system utilizing as a separating menstrum colloidal silica (Ludox AM®). The primary advantages of this preparation are: (1) It is chemically defined, allowing extremely reproducible cell separation employing different lots of material; (2) the physical parameters (pH, density, salt concentration) of the final gradient suspension can be manipulated over a wide range of values, allowing for the separation of many different biological materials; (3) it allows separation of very large numbers of lymphoid cells with greater than 95% recovery of applied cells; (4) separated cellular subpopulations can be easily washed free of silica and cellular function is retained. This paper is a report of the preparation and functional characteristics of the gradient material as it relates to the separation of very large numbers of lymphoid cell subpopulations in both mouse and man. Subpopulations of murine and human lymphocytes separated by this gradient material were assayed for IgM synthesis, T-cell mediated cytotoxicity, and lymphokine production.

INTRODUCTION

The successful use of density gradient separation of cells and organelles has played a major role in establishing functional relationships between cellular and subcellular systems. Cellular fractionation is of particular importance in the study of the immune response because of the heterogeneity of effector cells in both cell-mediated (Perlman and Holm, 1969; Granger, 1972) and humoral immunity (Mosier, 1967; Raidt et al., 1968). Various gradient media have been developed and used for the separation of various cells and organelles including lymphoid cell populations. These include bovine serum albumin (Raidt et al., 1968; Shortman et al., 1968; Durkin et al., 1975), Ficoll-sodium and meglumine 3,5-diacetamido-2,4,6-triiodobenzoic acid salts (Yu et al., 1974; Aspray et al., 1975; Dow and Pretlow, 1975; Böyum, 1976), silica gels (Pertoft et al., 1968; Lagercrantz et al., 1970; Pertoft, 1970; Morgenthaler et al., 1974), gum acacia (Sugiyama et al.,...
1977), gelatin (Coulson, 1964), and dextran (Hilal et al., 1964; Shenker and Gray, 1976). The two most widely used gradient preparations for the isolation and separation of lymphoid cell populations are bovine serum albumin and Ficoll-metrizoate salt solutions.

Bovine serum albumin, because it is a biological product, may demonstrate separation and yield variability from lot to lot (Shortman, 1968). Furthermore, because of its tendency to cause cell aggregation, BSA has forced some investigators to work at reduced pH (pH 5.1) (Shortman, 1968). Low pH may cause cells to swell, thus altering their gradient migration patterns (Gorczynski et al., 1970). It is also expensive to use in large scale separations. Even with these restrictions, BSA gradients have been extremely popular and useful. They have been employed by investigators to study subpopulations and interaction between subpopulations of lymphoid cells in both humoral (Raidt, 1968) and cell-mediated (Kelton et al., 1972) immunity. Ficoll may cause cellular aggregation, which requires the use of a dispersing agent (Gorczynski et al., 1970), lowering of the pH (Gorczynski et al., 1970), or the use of dispersing enzymes (Kelton et al., 1972), and it is highly viscous (Pretlow, 1973). In addition, it is difficult to produce a linear iso-osmotic gradient (Williams et al., 1972). Both materials suffer from the inability to separate very large numbers of functional subpopulations of lymphoid cells on convenient sized gradients with recoveries of greater than 90%.

Of the many techniques routinely employed to separate cells, isopycnic gradient centrifugation is one of the most practical. In addition to the ease with which it can be employed in many biological systems and its ability to be readily adapted to preparative experimental designs, it has a great degree of flexibility. This flexibility provides the investigator with the potential to separate many subpopulations of lymphoid cells by adjusting various physical parameters of the density gradient material. The first report of the use of colloidal silica for the separation of cells was by Mateyko and Kopac (1963). Pertof modified the original silica gel composition by adding polysaccharides to stabilize the gradient (Pertoft, 1966) and utilized discontinuous silica gel gradients for the separation of whole blood into four distinct populations consisting of red cells, polymorphonuclear cells, mononuclear cells, and thrombocytes (Pertoft et al., 1968; Evrin and Pertof, 1973). Although cell recovery was good (78–90%), a dipotassium salt of 2-napthol-6,8-disulfonic acid must be added to the Ludox HS to inhibit cell aggregation. Lymphocyte transformation, as assayed by DNA synthesis following PHA stimulation, was lost if reducing reagents were not incorporated into the gel (i.e., glutathione). A new formulation using silica gel AM is described which has the following useful characteristics: (1) reproducibility; (2) ease of preparation; (3) nontoxicity; (4) ability to separate very large numbers of lymphoid cells (5 × 10^8 cells/5 ml gradient material); (5) ability to separate cells into functionally intact and distinct subpopulations.

Data is presented which demonstrates the ability of this gradient prepara-
tion to separate large numbers of antibody synthesizing cells, antibody forming precursor cells, cytotoxic T-cells, and lymphokine secreting cells.

MATERIALS AND METHODS

Preparation of gradient material (Fig. 1)

Colloidal silica preparation. Aluminum modified silica, (Ludox AM®, Dupont, 1972) which comes as a 30% (w/w) suspension in distilled water, is first neutralized (pH 7.1) with 1.0 N HCl. It is then passed through a type A fiberglass filter (Gelman, Ann Arbor, Michigan) to remove any precipitate and large aggregates. The filtrate is dialyzed against glass distilled water, at a ratio of 1 vol against 20 vol for 3 days at 4°C with daily changes. This solution is then autoclaved at 121°C 15 lb for 15 min.

Gradient diluent preparation. Stock (5×) PVP-balanced salt solution is prepared in two component parts as follows:

Salt Solution 1: Dextrose, 500 mg; KH2PO4, 300 mg; Na2HPO4 • 7 H2O, 1740 mg; polyvinylpyrrolidone (Sigma Co. No. PVP-40), 8750 mg to 500 ml final volume glass distilled water.

Salt Solution 2: CaCl2 • 2 H2O, 930 mg; KCl, 2000 mg; NaCl, 40,000 mg; MgCl2 • 6 H2O, 1000 mg; MgSO4 • 7 H2O, 1000 mg; PVP-40, 8750 mg to 500 ml final volume distilled water.

Fig. 1. Gradient preparation schematic.
Equal volumes of Solution 1 and 2 are mixed to form Solution 3; the 5× stock PVP-BSS. This solution is adjusted to pH 7.1 with 1.0 N NaOH and filter sterilized.

**Preparation of working gradient medium**

An isotonic diluent stock solution (3.5% PVP-BSS-colloidal silica) is obtained by mixing one part 5× stock PVP-BSS (Solution 3) with 4 parts colloidal silica. This solution must mix for 2 h at room temperature. The density of this working solution may be adjusted with 1× PVP-BSS (obtained by adding 1 part 5× PVP-BSS (Solution 3) to 4 parts glass distilled water).

After extensive studies with a variety of various colloidal silica compounds and their effect on lymphocytes, Ludox AM (aluminum modified, Registered Trademark of E.I. Du Pont De Nemours and Co., 1972) was selected. This choice was based on: (1) its stability over a wide pH range; (2) its total innocuity to lymphoid cells; (3) it does not aggregate lymphoid cells; (4) the ease of gradients preparation; (5) and finally the ability of modified Ludox AM to separate large numbers of lymphocyte subpopulations.

**Density determination**

The density of each gradient component was determined using an organic solvent density column method described by Pertolt and Wolff (Pertoft, 1966; Wolff, 1975), using kerosene and carbon tetrachloride and sucrose standard density solutions. Stock solutions of Ludox AM gradient material were adjusted to densities of 1.050, 1.060, 1.070, 1.080, 1.090, and 1.100 gm/ml. The refractive index of each solution was determined using a refractometer (Bausch and Lomb No. 3-L) and a standard curve was generated for use in future density determinations. The density of each solution was also checked by weight before each experiment.

**Osmolarity determination**

Osmolarity was determined for each gradient density by measuring freezing point depression (Advanced Hi-Precision Osmometer model 3R) and vapor pressure determination (Wescor Osmometer, Model 5100).

**Storage of gradient material**. The Ludox AM gradient material may be stored at 4°C or room temperature for periods up to 5 years without detectable physical alteration. The gradient material cannot be frozen, however, because the colloid irreversibly precipitates at −1°C. The gradient material will support the growth of various micro-organisms and should be stored under sterile conditions. Streptomycin (100 µg/ml), penicillin (100 units/ml) and fungizone (2.5 µg/ml) are added when the gradient material is made to inhibit the growth of potential contaminating microorganisms which may be found in various lymphoid tissues (i.e., tonsil).

**Formation of gradients** (Fig. 2). The stock gradient solution was adjusted to various densities by adding varying amounts of 1× PVP-BSS. 1.0 ml
Fig. 2. Schematic representation of Ludox AM 5.0 ml gradient. Gradients were formed by sequential over-layering of 1.0 ml volumes of the following densities: 1.090, 1.080, 1.070, 1.060, 1.050 g/ml. Centrifugation was carried out at 4°C for 30 min and 1100 × g_{max}. Cells are distributed into distinct subpopulations at each density interface labeled A–E and were harvested via aspiration with a Pasteur pipette.

samples of densities 1.090, 1.080, 1.070, 1.060, and 1.050 were sequentially overlayered in 2'' × 1/2'' polyallomer centrifuge tubes (Beckman No. 326819). This was accomplished by carefully layering the less dense solutions on top of the more dense solutions with a pipette or syringe and needle. While we routinely use the overlayer procedure, the less dense solutions may be added first and then floated to the top by placing the denser solutions beneath them.

Isopycnic gradient centrifugation. Various numbers of lymphocytes up to 5.0 × 10^8 cells in 1.0 ml of Hank’s balanced salt solution (BSS) were placed on top of the last gradient aliquot (1.050). Gradients were centrifuged at 1100 × g_{max} for 20 min at 4°C. Following centrifugation, the cells were removed from the interfaces of each density with a Pasteur pipette, washed twice by sedimentation at 300 × g_{max} in BSS and resuspended in appropriate media for culture or assay.

Lymphoid cell isolation and culture

Murine lymphocytes. Two to 3-month-old male BDF₁, C57BL/6J, or DBA/2J mice were used in all experiments. Spleens were removed from mice killed by cervical dislocation and teased apart in Hank’s balanced salt solution (BSS). Cell aggregates were allowed to settle out in conical centrifuge tubes held in ice for 10 min. The supernatant containing mostly single cells was centrifuged at 4°C and 450 × g_{max} for 6 min. The packed cells were
resuspended in appropriate culture or assay media at various densities.  

**Human lymphocytes.** Tonsils, lymph nodes, and thymus tissue were obtained aseptically at the time of surgery and washed with cold Hank's balanced salt solution (BSS) containing streptomycin (100 µg/ml), penicillin (100 U/ml), and fungizone (2.5 µg/ml). The tissues were minced with scalpels in sterile Petri dishes containing BSS, transferred by pipette to culture tubes (Corning No. 25330), and allowed to stand in ice for 10 min. The supernatant containing single cells was removed and centrifuged at $450 \times g_{max}$ for 6 min at $4^\circ C$. The resultant cell pellets were resuspended in appropriate media for culture or assay. Viability was determined by eosin Y dye exclusion. Cell viability was routinely greater than 85%.

Peripheral blood lymphocytes were obtained by overlaying heparinized (preservative free, 100 U/ml) blood on Ficoll-Hypaque® density gradients, followed by centrifugation at $200 \times g_{max}$ for 40 min at $25^\circ C$. Interface cells were removed and washed 3 times in cold BSS and adjusted to appropriate density in culture or assay media.

**Adherent cell removal.** All lymphoid cell populations were incubated in 25 ml BSS at $37^\circ C$ for 30 min in 100 mm plastic dishes (Corning No. 25020 plastics) containing 10 g of 0.5 ml diameter glass beads. Following incubation, the nonadherent cells were removed with a Pasteur pipette and prepared for culture or separation. Determination of macrophage removal was accomplished using the ink technique described by Böyum (1968).

**Lymphocyte assays**

**IgM antisheep antibody production.** Mouse spleen cells recovered from the Ludox gradient were washed three times and resuspended in BSS to a density of $1.0 \times 10^7$ cells/ml. The number of plaque-forming cells was assayed by a modification of the technique described by Mishell (Mishell and Dutton, 1967). Briefly, 100 µl or 10 µl of spleen cell suspension was added to tubes containing 0.5 ml of 0.5% agarose in BSS at $44^\circ C$. 50 µl of an 8% sheep erythrocyte suspension was added to each tube and the contents of the tube was poured onto a microscope slide, overlayed with guinea pig complement ($1 : 15$ in BSS) and incubated for 3 h at $37^\circ C$. Hemolytic IgM plaques were counted over indirect light. Random plaques were examined microscopically to locate a single cell center.

**In vitro SRBC immunization.** In vitro stimulation of IgM antibody to sheep erythrocytes (SRBC) was conducted in a manner described by Mishell and Dutton (1967). Spleen cells isolated from the Ludox gradient were initiated in culture on day 0, immunized with $1 \times 10^6$ sheep erythrocytes on day 0 and assayed for IgM plaque forming cells on day 5 of culture.

**Lymphotoxin assay.** The assay for human lymphotoxin has been described elsewhere (Daynes and Granger, 1974). Briefly, alpha L-929 mouse fibroblasts were placed in $15 \times 120$ mm Kimax culture tubes in 1 ml of minimal essential media containing 3% newborn calf serum (heat inactivated, $56^\circ C$ for 30 min), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine,
and 50 μg/ml mitomycin C. Cells were incubated at 37°C with 5% CO\textsubscript{2} in air for 24 h. At the conclusion of the incubation period, the media in the test tubes was poured off and replaced with 1 ml of lymphotoxin (LT)-containing media at various dilutions. The tubes were incubated at 37°C for 24 h. The amount of LT in the supernatant was determined by counting the total number of cells remaining in the assay tubes with a Coulter Counter and comparing them with control tubes.

**Production and assay of cytotoxic T-lymphocytes.** Cytotoxic T-lymphocytes were produced and assayed for in a manner described by Brunner (Brunner et al., 1968). Male 12-week-old C57B/6J mice were immunized by one intraperitoneal injection of 3 × 10\textsuperscript{7} heat killed DBA/2 mastocytoma P-815-x2 cells and their spleens harvested 12 days later. Spleen cell suspensions were isolated as previously described and separated on the Ludox AM gradient. Each subpopulation was harvested and adjusted to 1 × 10\textsuperscript{7} cells/ml in MEM Eagles, and 1 ml was placed in 10 × 25 mm flat-bottomed tubes.

Cultured DBA mastocytoma cells were used as target cells in the assay. Target cells were labeled with \textsuperscript{51}Cr as described by Brunner et al. (1968). The labeled tumor target cells were resuspended in MEM Eagles to a concentration of 1 × 10\textsuperscript{6}/ml, and 0.1 ml of target cells was placed in 10 × 25 mm flat-bottomed tubes with 1.0 × 10\textsuperscript{7} spleen subpopulations. All tubes were incubated without rocking at 37°C in 5% CO\textsubscript{2} for 12 h. Following incubation, tubes were centrifuged at 1500 × g\textsubscript{max} for 15 min, and 0.8 ml of supernatant was carefully removed. The radioactivity of the supernatants was counted in a well type gamma counter (Beckman, Model 2000). The results were expressed as percent specific kill as calculated by the following formula:

\[
\frac{\text{\textsuperscript{51}Cr released with immunized spleen (counts)} - \text{\textsuperscript{51}Cr released with nonimmunized spleen (counts)}}{\text{Maximum \textsuperscript{51}Cr release (counts)} - \text{\textsuperscript{51}Cr released with non-immunized spleen (counts)}} \times 100 = \% \text{ Specific kill}
\]

Maximum \textsuperscript{51}Cr release was determined by freezing and thawing a 1.1 ml sample of \textsuperscript{51}Cr labeled cells 4 times and counting the supernatant.

**Preparation of gradient-separated cells for scanning electron microscopic evaluation (SEM)**

Preparation of human tonsil lymphocytes separated on Ludox AM gradient for SEM was done using the technique described by Polliack (1974). Briefly, washed cells were collected on 0.45 μm porosity silver membranes by filtration, fixed for 1 h with 1% glutaraldehyde, rinsed with phosphate buffer (pH 7.3, 310 mosmole), post fixed with 1% osmium tetroxide for 1 h, washed in buffer and dehydrated in alcohol for 5 min at each concentration. Complete dehydration was continued through absolute amyl acetate and each filter was dried with carbon dioxide at 900 lb/in.\textsuperscript{2} for 15 min. The membranes were coated with carbon and gold at an angle of 15°
and examined in a Cambridge 54 microscope at an accelerating voltage of 15–18 kV and 200 μm diameter illuminating aperture. Fifty fields for each subpopulation were photographed for evaluation.

RESULTS

Osmolarity and density

One major problem associated with density gradient cell separations is maintaining constant osmolarity throughout the gradient, so that cell size and viability are not altered during the separation process. Our studies determined that Ludox AM has a very minor contribution to osmolarity, and that its effect, as shown in Fig. 3, is linear. It is therefore a simple matter to construct density gradients which are iso-osmotic using this compounds.

In order to simplify the process of forming gradients of specific densities and to facilitate the determination of the densities of gradient material at which a specific cell type banded, the relationship of density vs. refractive index was examined. The density of the gradient material vs. refractive index, and the density of the gradient material vs. concentration (w/w) are compared in Fig. 4. Twelve different batches of gradient material from two different lots of Ludox AM were made using the refractive index as a determination of density. Each gradient solution was then tested for density using the organic solvent density column method. Solutions were found to be at the correct density ±0.5%. Refractive index is currently being used as the method of density adjustment. The discontinuous gradient was used as a model in developing the Ludox AM step gradient because of the clean separation of subpopulations of cells associated with step-type gradients, and also because of the past success that this type of gradient has enjoyed in separating functionally unique subpopulations of lymphoid cells. The gradients consisted of five different solutions of Ludox AM having densities

![Fig. 3. Plot of concentration (w/w) of Ludox AM colloidal silica versus osmolarity.](image-url)
Fig. 4. Relationship of Ludox AM concentration (w/w) and refractive index to density (g/ml).

of 1.050, 1.060, 1.070, 1.080, and 1.090 g/ml (Fig. 2). These densities were chosen because they are similar to those found useful in BSA gradients. The isosmotic nature of the gradient was demonstrated by measuring the osmolarity of each concentration. The osmolarity is constant throughout the gradient at the concentrations employed (Table 1).

Gradient capacity, yield, and reproducibility

Discontinuous gradients were poured in $\frac{1}{4}'' \times 2''$ polyallomer tubes (Beckman No. 326819) and loaded with 1.0 ml of BSS containing various numbers of human tonsil lymphocytes from $1 \times 10^4$ through $1 \times 10^{10}$ cells/ml. Following centrifugation, each of the five subpopulations were harvested by aspiration with Pasteur pipettes, counted via hemacytometer, and stained with 0.4%

### Table 1

OSMOLARITY DETERMINATIONS OF LUDOX AM-PVP GRADIENT MATERIAL AT THE CONCENTRATION EMPLOYED IN THE STANDARD STEP GRADIENT MEASURED BY BOTH VAPOR PRESSURE AND FREEZING POINT DEPRESSION

| % Ludox AM | Density (g/ml) | Osmolarity (mosM) |
|------------|----------------|-------------------|
|            | Vapor pressure | Freezing point    |
| 5.6        | 1.050          | 290.0             |
| 7.0        | 1.060          | 288.0             |
| 8.4        | 1.070          | 292.0             |
| 9.8        | 1.080          | 289.0             |
| 11.2       | 1.090          | 295.0             |
|            |                | 316.1             |
|            |                | 316.0             |
|            |                | 316.6             |
|            |                | 315.1             |
|            |                | 311.9             |
trypan blue (Gibco No. 525) for viability. Gradient capacity was defined as the maximum number of cells which were separated cleanly into distinct interfaces with greater than 90% recovery from the gradient. Maximum capacity for the 5 ml gradient was $5 \times 10^8$ lymphoid cells (Fig. 5). Both loss in recovery and interband smearing occurred at densities greater than $5 \times 10^8$ cells per gradient. Since recovery is one of the major problems associated with any cell separation procedure, direct cell counts were performed on gradient separated lymphoid cells from a variety of lymphoid tissues. Each tissue was dissociated in cold BSS and $5 \times 10^8$ cells were placed on 5 ml gradients. The cell recovery for mouse spleen, human peripheral blood, human tonsil, human lymph node, and human thymus cells were determined for two different lots of Ludox AM. Cell recovery was greater than 80% for all tissues tested with the single exception of human thymus lymphoid cells (77.3%) and cell viability was greater than 90% for all tissues tested (Table 2).

Ludox AM gradient separation reproducibility was investigated in several ways. The first method employed lymphocytes from mouse spleen, human peripheral blood, tonsil, lymph node, and thymus. The cells were separated on 5 ml gradients and each subpopulation was counted and expressed as the percentage of the total cells recovered from the gradient. Many different tissue samples were separated and the subpopulation distribution was found to be very reproducible within the same tissue type (Table 3). The second method employed human tonsil lymphocytes ($5 \times 10^8$ cells/5 ml gradient). These cells were separated and the subpopulations A, C, and E were

![Fig. 5. Ludox AM gradient capacity and yield. $5 \times 10^8$ human tonsil lymphocytes were applied to the top of 5 ml gradients in 1.0 ml BSS. Each point represents the average of 8 experiments ±1 S.D. Inter-band smearing percent was determined by counting the cells between the interfaces and expressing this as the percent of the total cells recovered from the gradient.](image)
TABLE 2
RECOVERY AND VIABILITY OF LYMPHOCYTES FROM VARIOUS LYMPHOID TISSUES FOLLOWING SEPARATION ON 5 ml LUDOX AM GRADIENTS

| Tissue type         | Ludox AM Lot No. | Experiment recovery (%) | Mean recovery (%) | Viability (%) |
|---------------------|------------------|-------------------------|-------------------|---------------|
| Mouse spleen        | 4 AR             | 99, 92, 91, 84          | 91.5              | 96            |
|                     | 5 AR             | 92, 88, 89, 99          | 89.5              | 91            |
| Human peripheral    | 4 AR             | 86, 84, 96, 97          | 90.8              | 97            |
| blood               | 5 AR             | 88, 83, 97, 90          | 89.5              | 94            |
| Human               | 4 AR             | 81, 92, 90, 90          | 88.2              | 90            |
| tonsil              | 5 AR             | 94, 91, 94, 88          | 91.8              | 93            |
| Human thymus        | 5 AR             | 94, 71, 67              | 77.3              | 94            |
| Human lymph node    | 5 AR             | 91, 90                  | 90.5              | 96            |

collected, washed and each subpopulation was applied to a new 5 ml gradient and separated. The number of cells at each density interface in these gradients were determined by direct count. The data shown in Fig. 6 indicated that greater than 85% of the reseparated cells in subpopulations A, C, and E

TABLE 3
CELL DISTRIBUTION AT EACH DENSITY INTERFACE EXPRESSED AS PERCENT OF TOTAL CELLS ISOLATED FROM 5 ml GRADIENT

| Gradient sub-population | Lymphoid tissue source       |
|-------------------------|------------------------------|
|                         | Mouse spleen                 |
|                         | Human peripheral blood       |
|                         | Human tonsil                 |
|                         | Human lymph node             |
|                         | Human thymus                 |
| A                       | 9.4 ± 1.6 a                  |
| (97) b                  | 6.1 ± 2.1                    |
| 24.2 ± 5.8              | 23.8 ± 5.1                   |
| (94)                    | 23.7 ± 5.0                   |
| C                       | 37.6 ± 6.2                   |
| (98)                    | 47.6 ± 9.8                   |
| D                       | 15.6 ± 3.1                   |
| (99)                    | 16.2 ± 4.6                   |
| E                       | 7.2 ± 2.1                    |
| (92)                    | 5.2 ± 3.1                    |
| P                       | 6.0 ± 3.0                    |
| (42)                    | 1.1 ± 1.0                    |
| N c                     | 30                           |

a Standard deviation.
b Viability.
c N = number of experiments.
were recovered in the appropriate density interface. The majority of cells which did not reseparate into the appropriate density were recovered in the pellet and were not viable.

**Effect of gradient separation of IgM secreting lymphocytes**

BDF$_1$ mice were injected with 0.2 ml (20% SRBC v/v) i.v. on day 0 and spleen lymphocytes were harvested 1, 3, and 5 days later for separation on 5 ml gradients. Cells recovered from the gradients were assayed for IgM antibody to SRBC and compared with the number of antibody producing cells in the unseparated spleen population. Gradient separation did not result in appreciable loss of antibody producing lymphocytes (Table 4).

The ability of the Ludox AM gradient to separate IgM secreting precursors was also investigated. BDF$_1$ spleen cells were separated on 5 ml gradients and $1 \times 10^7$ of each subpopulation was cultured in vitro with $1 \times 10^6$ SRBC for 5 days with supplementation of $1 \times 10^5$ adherant cells.

IgM plaque assays were run on each culture in triplicate. IgM secreting precursors were predominantly found in subpopulations B, C, and D with subpopulation C showing enrichment over the unseparated spleen and recombined spleen values (Table 5).

**Effect of gradient separation on cytotoxic lymphocytes**

C57B1/6J mice were immunized with $3 \times 10^7$ DBA/2 mastocytoma P-815-X2 cells intraperitoneally and 12 days later the lymphocytes from their spleens were isolated and separated on 5 ml gradients, washed and assayed.
TABLE 4
EFFECT OF LUDOX AM GRADIENT SEPARATION ON IN VIVO PRIMED IgM SECRETING CELLS

Mice were primed with SRBC at various times and their spleens separated and assayed for IgM plaque forming cells.

| Days post immunization | Number of antibody forming cells loaded on gradient | Number of antibody forming cells recovered from gradient | Recovery (%) | Mean |
|------------------------|------------------------------------------------------|---------------------------------------------------------|--------------|------|
| 5                      | 2.16 x 10^5                                         | 1.79 x 10^5                                             | 83           |      |
|                        | 1.08 x 10^5                                         | 1.05 x 10^5                                             | 97           | 91.3 |
|                        | 1.72 x 10^5                                         | 1.62 x 10^5                                             | 94           |      |
| 3                      | 1.12 x 10^4                                         | 1.01 x 10^4                                             | 90           |      |
|                        | 1.37 x 10^4                                         | 1.19 x 10^4                                             | 87           | 84.7 |
|                        | 1.22 x 10^4                                         | 9.4 x 10^3                                              | 77           |      |
| 1                      | 5.17 x 10^2                                         | 4.29 x 10^2                                             | 83           |      |
|                        | 3.72 x 10^2                                         | 2.84 x 10^2                                             | 76           | 86.0 |
|                        | 1.12 x 10^2                                         | 1.11 x 10^2                                             | 99           |      |

for specific cytotoxicity to ^51^Cr-labeled mastocytoma. Ludox gradient separation of tumor immune spleen resulted in a separation of cytotoxic lymphocytes into three subpopulations, C, D, and E. Subpopulation D was slightly enriched in cytotoxic lymphocytes (Table 6). Recombined spleen cells demonstrated a level of cytotoxicity equal to the unseparated population.

Effect of gradient separation on LT lymphokine production

Human tonsil lymphocytes were separated on 5 ml gradients, washed, and incubated for 72 h at 37°C in RPMI-1640 with 1.0 mg/ml PHA-P and the culture supernatants assayed for lymphotoxin production (LT). As can be

TABLE 5
EFFECT OF GRADIENT SEPARATION ON MOUSE IgM ANTIBODY FORMING PRECURSORS

Figures represent number of antibody-forming cells/10^6 recovered viable cells after 5 days of culture with SRBC antigen followed by gradient separation, wash, and assay.

| Unseparated spleen | Subpopulation |
|--------------------|---------------|
|                    | A  | B  | C  | D  | E  | P  | Recomb a |
| 6,747              | 96 | 1031 | 8227 | 1989 | 112 | 0  | 5,897 |
| 3,240              | 68 | 248  | 5841 | 1070 | 17  | 0  | 3,042 |
| 8,471              | 24 | 733  | 11,864| 1143 | 11  | 0  | 8,270 |

*Recomb = mouse spleen cells separated on gradient and remixed prior to culture.*
TABLE 6
EFFECT OF GRADIENT SEPARATION ON ALLOGENEIC CYTOTOXICITY
Figures represent percent specific kill as assayed by $^{51}$C release using a spleen cell to
target cell ratio of 100 : 1

| Unseparated spleen | Lymphocyte subpopulation |
|--------------------|--------------------------|
|                    | Band A | Band B | Band C | Band D | Band E | Recomb $^a$ |
| 61                 | 0      | 0      | 16     | 75     | 14     | 58         |
| 73                 | 0      | 0      | 14     | 81     | 6      | 78         |
| 81                 | 0      | 0      | 19     | 90     | 7      | 75         |
| 47                 | 0      | 0      | 6      | 66     | 11     | 40         |

$^a$ Recomb = mouse spleen cells separated on gradient and remixed before assay.

seen from the data in Table 7, gradient separation did not alter the lym-
photoxin titer. Gradient separation of human tonsil lymphocytes results in
the separation of subpopulations of lymphocytes which produced LT in
normal amounts (D and E) and subpopulations which produce decreasing
amounts of LT when compared to the unseparated tonsil population (popula-
tions A, B, and C). Subpopulation E (density >1.080) produces the highest
titers of LT but is rather non-reactive in the allogeneic cytotoxicity reaction.

SEM of gradient separated lymphocyte subpopulations

Human tonsil lymphocytes were separated on 5 ml gradients and prepared
for SEM examination. As can be seen in Fig. 7, most of the cells in sub-
populations A and B are large (>15 $\mu$m) and irregular in shape with many

TABLE 7
EFFECT OF GRADIENT SEPARATION OF HUMAN TONSIL ON LYMOPHTOXIN
PRODUCTION

| Unseparated tonsil | Band A | Band B | Band C | Band D | Band E | Recomb $^a$ |
|--------------------|--------|--------|--------|--------|--------|-------------|
| 6250               | 520    | 1096   | 4010   | 6000   | 7000   | 5990        |
| 4370               | 400    | 610    | 1081   | 3800   | 4160   | 4440        |
| 5040               | 350    | 410    | 770    | 4100   | 5260   | 5010        |
| 1070               | 90     | 180    | 470    | 680    | 800    | 1060        |

$^a$ Recomb = mouse spleen cells separated on gradient and remixed before assay.

$^b$ One unit is equal to the reciprocal lymphotoxin dilution which will cause a 50% reduct-
ion in the number of viable target cells after a 24 h incubation period when compared to
controls.
Fig. 7. Scanning electron microscope images of the subpopulations of tonsil lymphocytes separated by the Ludox AM gradient. Subpopulation U is from the pellet and contains mostly non-viable cells.

projections. Subpopulations C and E contain approximately 50% small (<15 μm) smooth lymphocytes and subpopulation D contains mostly small smooth lymphocytes. Each subpopulation is characterized by a unique mixture of size and surface morphology, although this is difficult to interpret functionally. The gradient separated subpopulations of lymphocytes were washed twice with BSS before preparation for SEM and this was sufficient to remove all gradient material (Fig. 7).

DISCUSSION

The results presented in this report demonstrate the usefulness of Ludox AM as a gradient material for the separation of large numbers of lymphoid cells from various tissues in both mouse and man. Gradient separation with Ludox AM has been shown not to impair antibody synthesizing cells, cytotoxic cells and lymphotoxin secretion. In addition, antibody secreting cells and cytotoxic precursor cells can be differentially separated from whole lymphocyte populations.
The major advantages of the Ludox AM gradient material are: (1) ease of preparation and low cost; (2) maintenance of physiological osmolarity and wide pH stability for densities useful in cell separation procedures; (3) very high cell load capacities not found with Ficoll-Hypaque, albumin, or metrizamide; (4) nontoxicity to lymphoid cell populations not found with gradients constructed with Ludox-HS; (5) very high recovery yields not found with other gradient materials; (6) lack of membrane penetration and ability to easily remove gradient material from cells with simple washes.

The separation of lymphoid cells which participate in mixed lymphocyte reactions, suppression of antibody synthesis, and syngeneic cytotoxicity to tumor antigens has also been completed and is the subject of another report.

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