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Use of Flow Cytometry to Quantify Mouse Gastric Epithelial Cell Populations

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Flow cytometry provides the opportunity to quantify cell populations within a total cell suspension. The quality of flow cytometry is strongly dependent on the isolation of intact viable cells. However, techniques to isolate mouse gastric cells for flow cytometry have not been evaluated. The objective of this study was to develop an effective method for isolating intact viable cells from mouse gastric tissue for flow cytometry. Cells were isolated from mouse stomach and spleen by either enzymatic separation or mechanical dissociation. A Percoll density gradient was used to separate viable cells from cellular debris. Cells were labeled with fluorescently tagged ligand or antibody and analyzed by flow cytometry. According to propidium iodide staining, there was a higher percentage of viable cells after mechanical dissociation (10–20%) compared to enzymatic separation (1%). After Percoll centrifugation there was a further increase in the percent of viable cells (50–80%). Gastrin (G), somatostatin (D), and parietal cells represented 0.6%, 3%, and 8% of the total epithelial cell population, respectively. T and B lymphocytes made up 4% and 2% in the gastric mucosa. Dissociated splenocytes were comprised of 20% T cells and 14% B cells. The ability to reliably resolve a cellular fraction that comprises only 0.6% of the input marks a substantial improvement over morphometric methods. Therefore, mechanical dissociation of the stomach followed by use of a Percoll gradient is the preferred method for isolating viable intact gastric epithelial cells for flow cytometry.

KEY WORDS: *Helicobacter pylori*; gastric epithelial cells; gastrin; somatostatin; parietal cells.

Helicobacter pylori is linked to a number of gastric conditions including gastritis, peptic ulcers, and gastric cancer (1). The pathophysiologic effects of *H. pylori* on the gastric mucosa have been studied by immunohistochemistry, microscopy, and morphometry (2–4). Immunohistochemical analysis of tissue sections is the standard technique to analyze morphologic changes of cells. However, morphometry is required to quantify the changes observed. To enumerate various cell types requires counting many cells in multiple tissue sections to achieve statistical significance (2, 3, 5). However, flow cytometry provides the opportunity to quantify specific cell types within a cell suspension derived from the entire organ. Analysis by flow cytometry is limited only by the specificity of the antibodies and the quality of the isolated cells.

The principle of flow cytometry is based on identifying cell surface membrane antigens in cell populations with similar light-scattering properties, using antibodies conjugated to fluorochromes, for example, fluorescein isothiocyanate (FITC) (6, 7). Light scattering is used to differentiate cells based on size, shape, or internal complexity (granularity). Subpopulations of cells are separated from the total cell suspension by combining forward light scatter (cell size) and right-angle light scatter (cell shape or complexity) (6). The quality of data is strongly dependent on the effective isolation of viable cells. Furthermore, the

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identification of cell surface membrane antigens requires the preservation of plasma membranes (8). The majority of flow cytometric analysis involves the preparation of isolated nuclei for DNA staining (8, 9) or the isolation of lymphocytes for immunophenotyping of hematopoietic malignancies (7). However, studies comparing dissociation techniques for the isolation of intact, viable cells from tissue are difficult to find. Moreover, using flow cytometry to quantify cell populations from the gastrointestinal tract is rare, perhaps due to poor dissociation techniques and the multiple cell types to identify.

Flow cytometry does not distinguish between fluorescently labeled cellular debris and stained single cells. Therefore, the production of viable, single-cell suspensions is a requirement for reliable, accurate, reproducible analysis. The two common strategies used for dissociating cells from tissue are enzymatic and mechanical separation. The enzymes pronase, collagenase, and trypsin are often used for enzymatic dissociation of gastric mucosal cells (10–14). Although pronase effectively dissociates gastric epithelial cells (13, 14), variations in tissue, temperature, and enzyme concentration can result in a loss of pronase activity that subsequently leads to decreased cell viability and yield due to extended incubation time (8, 11). In general, mechanical dissociation methods are variable and include scraping, mincing, and needle aspiration (8, 15, 16).

Thus the aim of the present study was to develop an effective method for isolating mouse gastric epithelial cells for flow cytometry. Intact cell dissociation was assessed by the following parameters: overall yield of viable cells, the quality of the cell preparation for flow cytometry, and the identification of cell surface membrane antigens. We focused on labeling major phenotypic cell types in the stomach, which included gastrin (G), somatostatin (D), and parietal cells. The presence of T and B lymphocytes in the gastric mucosa was also analyzed in anticipation of applying flow cytometric analysis to the study of inflammatory disorders of the stomach, for example, *H. pylori* infection.

MATERIALS AND METHODS

Reagents

Bombesin, [Lys3]-bombesin 14 (Bachem California, Torance, California), was used to label G cells. [Lys3]-Bombesin 14 was conjugated to the fluorescent dye Cy3 (Amersham Pharmacia, Arlington Heights, Illinois) (1:100 dilution). The Kirsten sarcoma virus-transformed normal rat kidney (KNRK) cell line transformed with the rat GRP receptor (KNRK GRPr) was a kind gift from Drs. L. Slice and J. Walsh (UCLA). Rabbit anti-somatostatin (1:100 dilution) (Zymed Laboratories, San Francisco, California) was used to label D cells. Rabbit anti-somatostatin primary antibody was detected with a fluorescein isothiocyanate (FITC) -conjugated goat anti-rabbit IgG (1:200 dilution) (Vector Laboratories, Burlingame, California). Parietal cells were surface labeled with a 1:200 dilution of either anti-mouse proton pump antibody 1H9 to detect the α subunit of mouse H^+,K^+-ATPase or 2B6 to detect the β subunit of mouse H^+,K^+-ATPase (Medical & Biological Laboratories Co., Nagoya, Japan). The primary antibodies for mouse H^+,K^+-ATPase were detected with FITC-conjugated rat anti-mouse IgG1 (Cappel, ICN Pharmaceuticals, Aurora, Ohio). The total population of gastric epithelial cells was detected using rabbit anti-EGF receptor antibody (Santa Cruz Biotechnology, Santa Cruz, California) (1:200 dilution). Rabbit anti-EGF receptor primary antibody was detected using phycoerythrin (PE) -conjugated goat anti-rabbit IgG (1:200 dilution) (Vector). Isolated T and B cells were labeled with a 1:100 dilution of monoclonal antibodies to CD3 and CD19, respectively (PharMingen International, San Diego, California). FITC-conjugated rat anti-mouse IgG_{2b} (PharMingen) and mouse anti-rat IgG2a (PharMingen) were used as isotype controls for CD3 and CD19 staining. Propidium iodide (Sigma Chemical Co., Missouri) was used to stain prefixed permeabilized cells. Propidium iodide (PI) cannot penetrate viable intact cells and therefore was used as a measure of cells damaged during the isolation procedure. PI was used at a concentration of 1 μg/ml. The wash buffer was prepared in Hanks' balanced salt solution (Gibco BRL) with 0.1% bovine serum albumin (BSA). The dissociated cells were separated from debris on a 50% Percoll (Pharmacia, Uppsala, Sweden) gradient.

Animals

Wild-type mice (C57BL/6 $\times$ SJL, 3–4 months old) were fasted overnight and killed by inhalation of metofane (methoxyflurane, Mallinckrodt Veterinary, Inc.). The stomach and spleen were quickly excised, washed in PBS (4°C), and transferred to cold wash buffer. The stomach was opened along the greater curvature and rinsed with wash buffer. Stomach and spleen tissues were weighed and cells were dissociated by either enzymatic or mechanical methods.

Dissociation of Cells from Stomach and Spleen

Enzymatic Dissociation. Enzymatic dissociation of gastric cells was performed by incubating the whole stomach in 1% pronase dissolved in 0.9% saline for 1.5 hr at 37°C. The solution was removed and replaced with fresh solution every 30 min. The cells were then treated with DNase I at 37°C for 30 min, washed with cold buffer, and centrifuged at 3000 rpm for 5 min at 4°C. The cells were filtered through a 40-μm nylon cell mesh. Viability and yield were assessed by trypan blue exclusion. Approximately 5×10^6 cells were analyzed by flow cytometry.

Mechanical Dissociation Without Percoll Centrifugation Gradient. For mechanical dissociation, the stomach and spleen were agitated in the Medimachine (Dako Corpora-

tion, Carpinteria, California) using the Medicon blades (25KGY-2,5M.RAD) for 3 min. Cells were then passed through 50-μm Filcon filters (Dako). Viability and yield were again assessed by trypan blue exclusion. Approximately 5×10^6 cells were analyzed by flow cytometry.

Mechanical Dissociation with Percoll Gradient. In a separate experiment, a Percoll centrifugation gradient was used to increase the concentration of viable cells isolated by the mechanical method. Stomach and spleen tissues were agitated in the Medimachine. After filtration, the cells were centrifuged at 3000 rpm for 5 min at 4°C, the supernatant was discarded, and the cells were resuspended in 10 ml of buffer. A Percoll gradient was prepared by mixing 20 ml Percoll with 20 ml wash buffer and centrifuging at 14,000 rpm for 25 min at 25°C to form the gradient. Five milliliters of the resuspended cells were gently layered on top of the gradient and centrifuged at 2000 rpm for 25 min at 25°C.

After centrifugation, two layers were visible—the top layer containing the cell debris and a second layer containing viable cells that were subsequently collected using a needle and syringe to avoid disturbing the layers. Buffer (10 ml) was added to the cells and centrifuged at 3000 rpm for 5 min. The resulting cell pellet was resuspended in 3 ml of buffer. Viability and yield were assessed by trypan blue exclusion and approximately 1×10^7 cells were analyzed by flow cytometry.

Flow Cytometry

Aliquoted cells (5×10^6 or 1×10^7 cells/tube) were centrifuged at 3000 rpm for 5 min and resuspended in 100 μl of wash buffer. All staining reactions were performed at 4°C in the dark. G cells were stained with a [Lys3]-bombesin 14–Cy3 conjugate that was prepared by incubating the peptide with the Cy3 dye at room temperature for 30 min. The [Lys3]-bombesin 14–Cy3 was prepared according to the instructions accompanying the Cy3 labeling kit (Amersham Pharmacia). Labeled peptide was separated from unconjugated dye by reverse-phase HPLC (17). The specificity of [Lys3]-bombesin 14–Cy3 was verified using Kirsten sarcoma virus-transformed normal rat kidney (KNRK) cells transfected with the rat GRP receptor (17). KNRK and gastric G-cells were stained with [Lys3]-bombesin 14–Cy3 for 20 min and washed. KNRK cells were then analyzed flow cytometrically. An aliquot of unfractionated gastric cells was incubated with rabbit anti-EGF receptor primary antibody, followed by an additional 20 min incubation using FITC-conjugated goat anti-rabbit IgG. To label gastric epithelial cells, the above population was incubated for 20 min with the rabbit anti-EGF receptor antibody, washed, and incubated again for 20 min with FITC-conjugated anti-mouse IgG1 secondary antibody. To stain D cells, EGF labeled cells were permeabilized using the CytoStain Kit (Cytofix/Cytoperm) (PharMingen) prior to labeling with anti-somatostatin antibody. The cells were fixed and permeabilized for 20 min at 4°C, then washed twice with the wash solution. The permeabilized cell population was incubated with rabbit anti-human somatostatin antibody for 20 min, washed, and incubated for an additional 20 min with PE-conjugated goat anti-rabbit IgG. Parietal cells were stained by incubating samples with anti-mouse antibodies raised against either the α or β subunit of mouse H^+,K^+-ATPase followed by incubation with a FITC-conjugated anti-mouse IgG_1 secondary antibody. To double-label gastric epithelial cells, the parietal cell population was incubated for 20 min with the rabbit anti-EGF receptor antibody, washed, and incubated for an additional 20 min with PE-conjugated goat anti-rabbit IgG. Isolated T and B cells were labeled with FITC-conjugated anti-mouse CD3 and CD19, respectively. FITC-conjugated anti-rat IgG_{2b} and IgG_{2a} antibodies were used as isotype controls for T and B cells, respectively. Stained cells were analyzed by flow cytometry. To assess viability, cells were stained with PI for 2 min, washed, and then resuspended in buffer for flow cytometry analysis.

Cell surface fluorescence intensity was analyzed using a Coulter Elite ESP Cell Sorter (Beckman-Coulter Electronics, Hialeah, Florida). The cells (5×10^3 or 1×10^4) were acquired by list mode, and the results were displayed as either a single- or double-parameter histogram. A single-parameter histogram represents the frequency distribution of the cell number relative to the intensity of the signal. The double-parameter histogram depicts a subpopulation of cells based on the forward (cell size) and right-angle (cell shape or complexity) light-scatter properties.

Table 1. Yield of Viable Cells from Stomach and Spleen

Method	*Cells (per ml)*	*Viability (%)*
Stomach		
Enzymatic separation	1.30×10^6	1
Mechanical separation	1.06×10^{11}	10–20
Mechanical plus Percoll	1.02×10^6	50–60
Spleen		
Enzymatic separation	NT*	NT
Mechanical separation	3.66×10^{11}	70–80
Mechanical plus Percoll	7.62×10^{10}	70–80

* NT = not tested.

RESULTS

Dissociation of Cells from Stomach and Spleen: Enzymatic Versus Mechanical Dissociation. Table 1 compares the number of cells per milliliter isolated by enzymatic and mechanical dissociation. After mechanical dissociation there were approximately 1.1×10^{11} stomach cells/ml isolated compared to 1.3×10^6 stomach cells/ml after enzymatic separation. The viability, assessed by PI staining, showed that there was a 10 to 20-fold increase in cell viability after mechanical separation compared to enzymatic dissociation (Table 1). Figure 1 compares the double-parameter histograms for enzymatic and mechanical dissociation of gastric cells. After enzymatic dissociation, there was no indication of a subpopulation of cells in region A of the double-parameter histogram (Figure 1A) after staining with PI. After mechanical dissociation, a subpopulation of cells was found in region A of the double-parameter histogram with 10% staining for viable cells (Figure 1B). The epithelial cell population

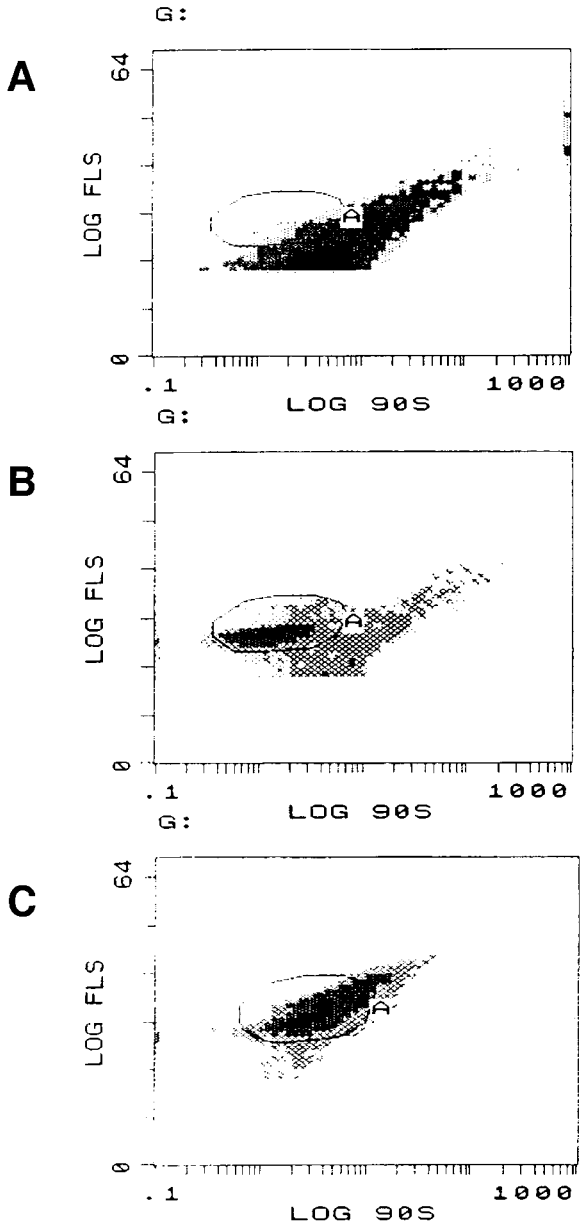

Fig 1. Double-parameter histograms of (A) enzymatic and (B) mechanical separation of cells from mouse stomach tissue, and (C) mechanical separation of mouse spleen cells. LOG FLS: forward light scatter (cell size), LOG 90S: right-angle light scatter (cell shape). Viable cell population is outlined in region A.

was identified using the antibody for the EGF receptor/IgG-FITC, where 92% of the cells in region A stained positive (Figure 1B). Figure 1C illustrates a clear subpopulation of spleen cells isolated after mechanical dissociation that did not stain positive for the antibody to the EGF receptor/IgG-FITC. There were more cells isolated from spleen (3.7×10^{11} cells/ml) after mechanical dissociation compared to stomach (Table 1).

Percoll Centrifugation Gradient. After mechanically separated cells were purified with a Percoll centrifugation gradient, there was a significant decrease in the number of gastric cells from 1.1×10^{11} to 1.0×10^{6} (Table 1). However, the viability of the remaining cells increased to 50–60%. Percoll purification of spleen cells after mechanical dissociation decreased the number of cells from 3.7×10^{11} cells/ml to 7.6×10^{10} cells/ml and the viability remained at 70–80% (Table 1).

Staining for G, D, Parietal, T, and B Cells. On a double-parameter histogram, cultured KNRK cells appeared as a clean population of cells in region A (Figure 2A). Flow cytometric analysis revealed that KNRK cells not transformed with the GRP receptor had 0% positive staining with [Lys3]-bombesin 14–Cy3 (Figure 2B). However, KNRK cells transformed with the GRP receptor showed 91% positive staining (Figure 2C). In a mixed population of KNRK cells and KNRK cells transformed with the GRP receptor (1:1), 41% cells stained with [Lys3]-bombesin 14–Cy3 (Figure 2D).

Figure 3 is a representative double-parameter histogram showing the average number of positively stained cells from stomach. Triplicate determinations performed on gastric epithelial cells isolated from mouse stomach revealed 56 ± 13 and 300 ± 21 G and D cells, respectively (Figures 3A and B). From 10,000 events that were counted, the percentage of positively stained G and D cells corresponded to 0.6% and 3%, respectively (Table 2). There was a difference in the number of stained parietal cells that depended upon the antibody type used. The H^+,K^+-ATPase antibody specific for the α subunit stained 3052 ± 1567 of the parietal cells, whereas the antibody for the β subunit stained 798 ± 52 of the cells (Figures 3C and D); These numbers corresponded to 31% and 8% of cells staining positive with the antibody for the α and β subunits, respectively (Table 2). There were more positively stained T and B cells in the spleen (2004 ± 834 and 1351 ± 219, respectively) compared to the stomach (449 ± 61 and 206 ± 48, respectively). From the 10,000 events that were counted, the percentage of positively stained T and B cells in the spleen was 20% and 14%, respectively (Table 2). T and B cells represented 4% and 2%, respectively, of the 5000 events counted from stomach cells (Table 2).

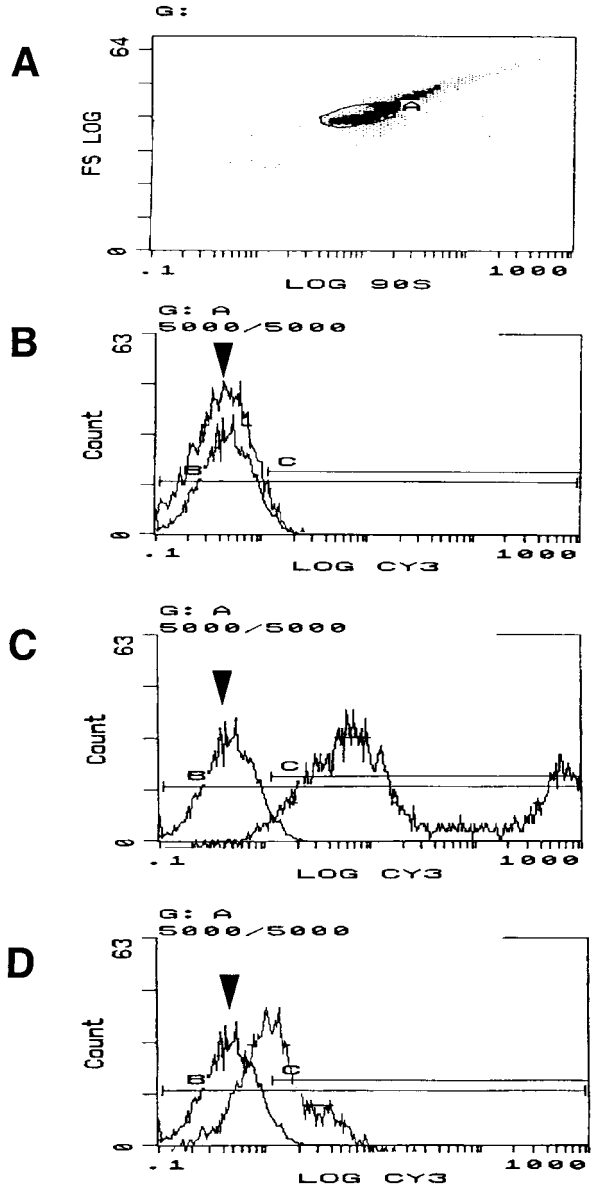

Fig 2. Single-parameter histograms of (A) rat Kirsten sacrcoma virus-transformed normal rat kidney (KNRK) cells, (B) stained KNRK cells, (C) stained KNRK (GRPr) cells, and (D) stained KNRK–KNRK (GRPr) cells (1:1). In B–D, letter B: total cell population, and letter C: positively stained cells. Viable cell population is outlined in region A. Unstained cells are indicated by the arrow.

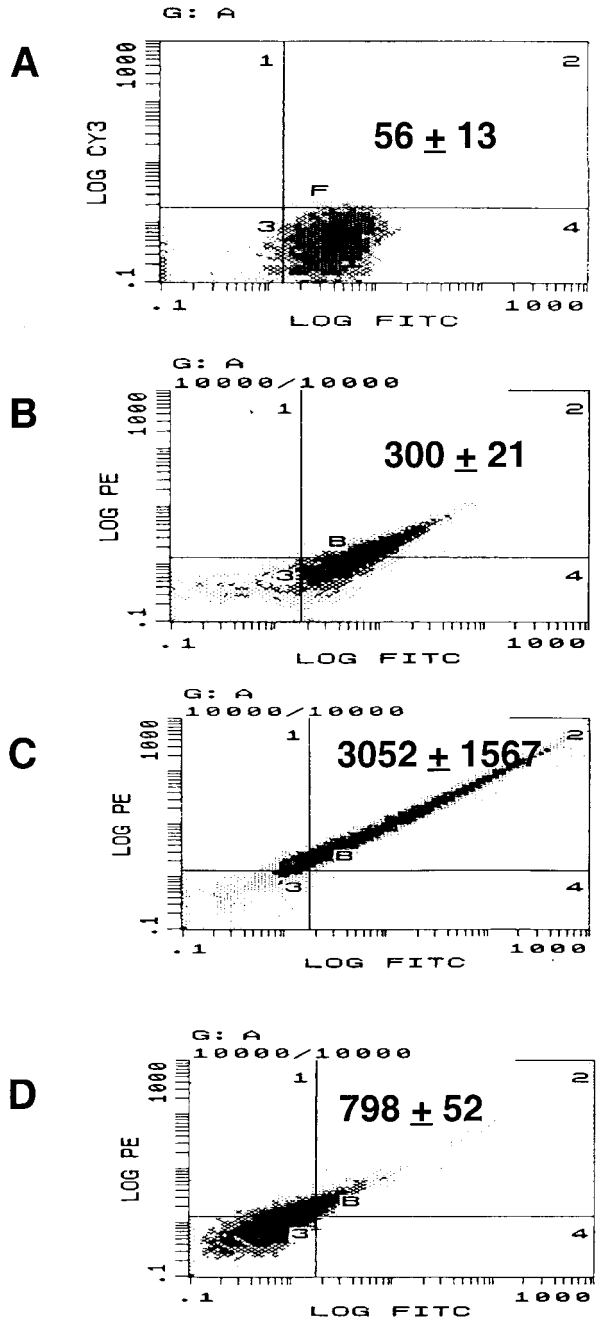

Fig 3. Histograms of mouse gastric cells stained for (A) gastrin, (B) somatostatin, (C) H^+,K^+-ATPase α subunit, and (D) H^+,K^+-ATPase β subunit. 1: positively stained Cy3 cells or PE, 2: positively stained Cy3 or PE plus FITC cells, 3: negatively stained cells, 4: positively stained FITC cells.

DISCUSSION

Although morphometric analysis is a standard method to quantify multiple populations of epithelial cells, it is a tedious and time-consuming method that relies heavily upon the accuracy of tissue preparation, sectioning, sampling, and statistical analysis. Alternatively, flow cytometry can be used to quantify cell populations within a total suspension. To develop a model for *H. pylori* pathogenesis, we have concentrated our efforts on applying flow cytometric analysis to mouse stomach. An important difference between large mammals and mice in applying flow cytometry

TABLE 2. POSITIVELY STAINED MOUSE GASTRIC CELLS

	*Percent stained cells (n = 3)**					
					H^+,K^+-ATPase	
Tissue	*Gastrin*	*Somatostatin*	*CD3*	*CD19*	*α subunit*	*β subunit*
Stomach	0.6	3	4	2	31	8
Spleen	NP†	NP	20	14	NP	NP

* Percent of positively stained cells from total cell population (approx. 10,000 cells counted).
† NP = no positive staining.

was the method of cellular dissociation. We report in this study a method for isolating mouse gastric epithelial and spleen cells by mechanical dissociation. Whereas enzymatic dissociation works well on gastric tissue from dogs and rabbits, where the starting material is abundant, this method resulted in substantial damage to the cells isolated from the mouse stomach.

After mechanical dissociation there was a significant increase in the number of isolated cells compared to enzymatic separation. In addition, the percentage of viable cells increased with mechanical separation. Some reports comparing mechanical and enzymatic dissociation of glandular tumors from colon show that, with respect to overall cell yield, neither approach is clearly superior (8, 18–20). Enzymatic digestion using proteases such as pronase and trypsin can often have damaging effects on cell surface membranes (8). Dissociation of cells from rat stomach by pronase is effective, but causes cell damage and loss of viability soon after separation (11). Another problem commonly encountered by enzymatic dissociation is enzyme sensitivity to time, temperature, and concentration. Variability in these factors can result in decreased cell yield and viability (8, 11). In another study, postseparation viability decreased with mechanical dissociation due to cytoplasmic trauma caused by shearing and tearing (21). Results may depend on the tissue type, source, and use of specific enzyme or mechanical dissociation techniques. Our results clearly show that mechanical dissociation is the preferred method for isolating gastric epithelial and spleen cells from mice.

Viable cells isolated by mechanical dissociation were separated from cell debris using Percoll centrifugation gradient. After Percoll centrifugation, there was a significant loss in the total number of cells. However, most of the recovered cells were viable due to the separation from lysed cells. There was also a loss of spleen cells during Percoll centrifugation, but the viability did not change, indicating that most of the cells recovered after mechanical dissociation were viable. Structural differences between stomach and spleen explain the difference in the number of cells isolated. There is more connective tissue and muscle in the stomach, as well as other parts of the luminal gastrointestinal tract, compared to parenchymal tissues such as the spleen and liver. Ficoll separation or micropore filtering following dissociation is recommended for maintaining cytometric components and removing macroscopic tissue aggregates (8, 22) Furthermore, density gradient separation using Percoll has been shown to be a useful method for purifying gastric epithelial cells from cellular debris and does not result in differential loss of one cell type over another (13).

After mechanical dissociation and Percoll purification, there were more cells stained with propidium iodide (PI) from the stomach than the spleen. PI has a similar chemical structure to ethidium bromide that intercalates between base pairs of double-stranded DNA (8). PI does not penetrate viable cells; thus exclusion of PI is often used to differentiate and quantitate viable and dead cells in an isolated population (8). In the present study there was a threefold increase in viable gastric cells not stained with PI after Percoll purification.

The G cells comprised 0.6% of the total population of epithelial cells. This agrees with flow cytometric analysis, which revealed approximately 1–2% of the cell population had high fluorescence intensity for [Lys3]-bombesin–Cy3 prior to enrichment (17). Furthermore, in the canine antrum, isolated G cells accounted for less than 1% of the total number of cells (23), and D cells comprised 3% of the total epithelial cell population. Other investigators have shown by immunocytochemistry that the relative frequency of D cells in the rat and human oxyntic mucosa is 10% and 26%, respectively (24, 25). D cells isolated from canine antral mucosa accounted for 1–2% of the total number of cells (23). Therefore, the relative frequency of different endocrine cells including D cells, varies among species (reviewed by Sundler and Hakanson; 26). Moreover, it is anticipated that there may be differences between D-cell numbers in the fundus versus the antrum.

From the total cell population identified as parietal cells, there was a higher percentage of positively stained cells with the antibody specific for the H^+,K^+-ATPase α subunit compared to that for the β subunit. The discrepancy in staining may be explained by the fact that of the two subunits, the b subunit is more specific for the H^+,K^+-ATPase. The α catalytic sub-

unit of the H^+,K^+-ATPase has 60% structural homology with the Na^+,K^+-ATPase that coexist on parietal cells of the gastric mucosa (27, 28). However, the β catalytic subunit of the H^+,K^+-ATPase shares only approximately 37% homology with that of the Na^+,K^+-ATPase (MacVector version 6.5, Oxford Molecular Group, Campbell, California). Therefore, using the antibody for the β subunit would give a more specific representation of the parietal cell population. Indeed, we found that by using the antibody to the β subunit, the number of parietal cells in the total gastric epithelial cells suspension was 8%. In support of this, Soll (14) found that in crude cell suspension isolated from canine gastric mucosa, parietal cells comprised less than 10%.

We conclude that mechanical dissociation is an effective method for isolating mouse gastric epithelial and spleen cells. Using flow cytometry, subpopulations of G, D, parietal, T, and B cells can be identified and quantified even when they comprise a small fraction of the original cell population. Therefore, this method can be used to quantify changes in the gastric mucosa in response to pathogenic conditions such as *Helicobacter pylori* infection.

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