

Subpopulation of Human Tonsillar Lymphocytes With Mitochondria Visible by Light Microscopy

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We observed a subpopulation of human tonsillar lymphocytes with cytoplasmic inclusions that stained orthochromatically blue with Wright stain. These lymphocytes represented fewer than 2% of cells in suspensions of cells from most tonsils; however, suspensions of tonsillar cells from 4 of 18 studied patients contained more than 2% (2.8%–6.4%) lymphocytes

with cytoplasmic inclusions. These cells sedimented less rapidly than the modal population of lymphocytes in a previously described isokinetic gradient. Histochemical and ultrastructural studies suggested that the stained inclusions were prominent mitochondria stained by the Wright stain.

WE REPORTED in 1975 a method for the separation of lymphocytes and plasma cells from the human tonsil.¹ In subsequent work with tonsillar cells, we found that the kinds of cells that we obtained in suspension from the human tonsil depended upon the method used to obtain the cells in suspension.^{2,3} In our early work with tonsillar cells in suspension, we noted a small proportion of lymphocytes with cytoplasmic inclusions that stained with Wright stain (LWCI). When these cells were fewer than 1% of the total cells from the tonsil, they could easily be ignored. More recently, we encountered a patient whose suspended tonsillar cells contained 6.4% LWCI as compared with 64.7% lymphocytes without inclusions (the remaining cells were erythrocytes, plasma cells, macrophages, etc.). More interestingly, we found that LWCI sedimented as a discrete subpopulation of lymphocytes in the previously described isokinetic gradient⁴ and that the purest fraction of these cells from one patient contained 31.2% LWCI. Because we had never encountered lymphocytes like these, we showed our slides to several experts on lymphocytes and were surprised to find that they were not familiar with these cells.

In this report we describe the frequency and sedimentation pattern of these LWCI. Histochemically, the distributions of the LWCI were similar to the distributions of cells with cytoplasmic inclusions with histochemically demonstrable succinic dehydrogenase. Ultrastructurally, gradient fractions that contained concentrated LWCI also contained lymphocytes with mitochondria larger than the mitochondria in the lymphocytes in fractions from the same gradients that contained no LWCI. We believe that the inclusions we observed

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in a subpopulation of human tonsillar lymphocytes are large mitochondria that stain an orthochromatic blue with Wright stain.

MATERIALS AND METHODS

Many of the tonsillar lymphocytes that we studied with Wright stain had been separated previously in isokinetic gradients for other studies.^{1,3} Our methods for obtaining these cells in suspension with 0.25% trypsin were described previously.^{1,2} The construction, centrifugation, and fractionation of density gradients were described previously.¹ Differential cell counts were performed by counting at least 500 cells/fraction as prepared for microscopic examination with a Cytocentrifuge (Shandon Southern Instruments, Sewickley, Pa.). The cells in each fraction were counted with hemocytometer chambers. The graphed distributions of cells in the gradients were calculated from the hemocytometer counts and the differential counts of cytocentrifuge preparations.

In order to characterize the cytoplasmic inclusions histochemically, separated cells were stained with alcian blue-safranin at pH 1.0 with the technique of Spicer⁵ as modified by Combs et al.⁶ and described in detail in our previous application of this technique.⁷ For the demonstration of acid phosphatase, separated cells were fixed in 10% formalin and 1% CaCl₂ for 1 min, stained for 2 hr with the technique of Burstone,⁸ and counterstained with 1% methyl green as described by our laboratory previously.⁹ Separated cells were stained for catalase at pH 9.0 by a modification of the technique of Novikoff and Goldfischer¹⁰ after fixation in 4% formalin with 1% glutaraldehyde (pH 7.2) for 1 min at room temperature; they were counterstained with 1% methyl green. For the demonstration of succinic dehydrogenase, separated cells were fixed with 10% formalin with 1% CaCl₂ and stained for 2 hr with the method described by Melnick.¹¹ The color formed by the reaction for succinic dehydrogenase faded appreciably after 3-4 days, and differential cell counts were always carried out within 24 hr of the separation of cells. In preliminary studies, cells were stained supravivally with Janus green B with the method as presented by Lillie and Fullmer.¹² When counts were performed rapidly after staining with Janus green B, they agreed closely with counts of cells stained for succinic dehydrogenase. While staining for succinic dehydrogenase was more time consuming than staining with Janus green B, the stability of the stain for succinic dehydrogenase permitted the preparations to be counted independently by more than one observer and led us to select succinic dehydrogenase for the investigations to be reported.

For electron microscopy, cells were sedimented to form a pellet, fixed in 4% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4 for 1 hr at room temperature, washed with PBS, stored overnight at 4°C in PBS, fixed in 2% OsO₄ in PBS for 30 min at room temperature, dehydrated in graded alcohols, and embedded in Spurr epoxy resin (Polysciences, Warrington, Pa.). Sections for electron microscopy were cut with a diamond knife (DuPont, Wilmington, Del.), doubly stained with lead citrate and uranyl magnesium acetate, and examined in a Philips EM-200 electron microscope.

RESULTS

The suspensions of unseparated tonsillar cells from 18 patients were studied. Suspensions from 4 patients contained 6.4%, 4.8%, 4.0%, and 2.8% LWCI; the suspensions of tonsillar cells from the remaining 14 patients all contained fewer than 2% LWCI. After velocity sedimentation in the isokinetic gradient for 16 min at 97 g, the modal population of LWCI was found in fraction 7 (± 1 , 4-ml fraction) from the isokinetic gradient (Fig. 1). These cells were purest in fractions 5-7; they were four to fivefold purified over those in the unseparated suspensions of tonsillar cells. In contrast, the modal population of lymphocytes was located in fraction 9 (± 1 , 4-ml fraction). As reported previously,¹ lymphocytes were 93.6% \pm 1.9% of nucleated cells and 91.6% \pm 1.9% of all cells in the purified fractions.

Immature mast cells have cytoplasmic inclusions and may bear a resemblance

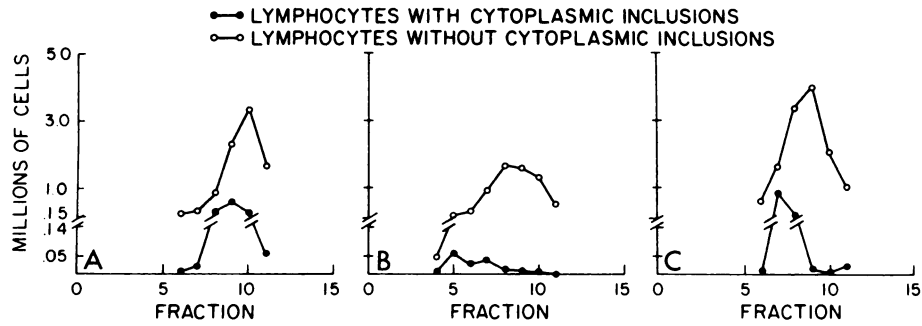


Fig. 1. Representative distributions of human tonsillar cells from three patients after sedimentation in isokinetic gradient for 16 min at 4°C at 97 g (measured at sample-gradient interface). Modal population of all lymphocytes sedimented more rapidly than modal population of lymphocytes with cytoplasmic inclusions that stained with Wright stain.

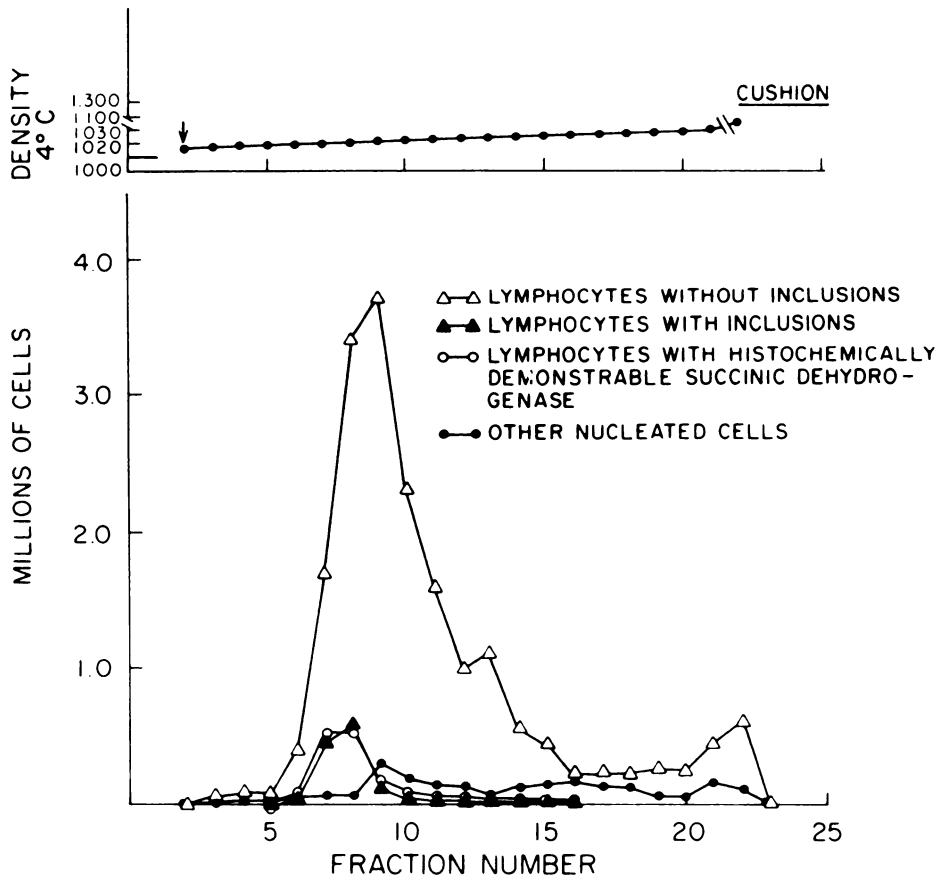


Fig. 2. Distribution of cells with cytoplasmic inclusions stained with Wright stain and cells with cytoplasmic inclusions with histochemically demonstrable succinic dehydrogenase. Cells from this tonsil sedimented similarly to those from several other tonsils studied both with Wright stain and with histochemical stain for succinic dehydrogenase. Close correspondence between these two markers suggested that cytoplasmic inclusions might be mitochondria. Arrow, sample-gradient interface on density plot.

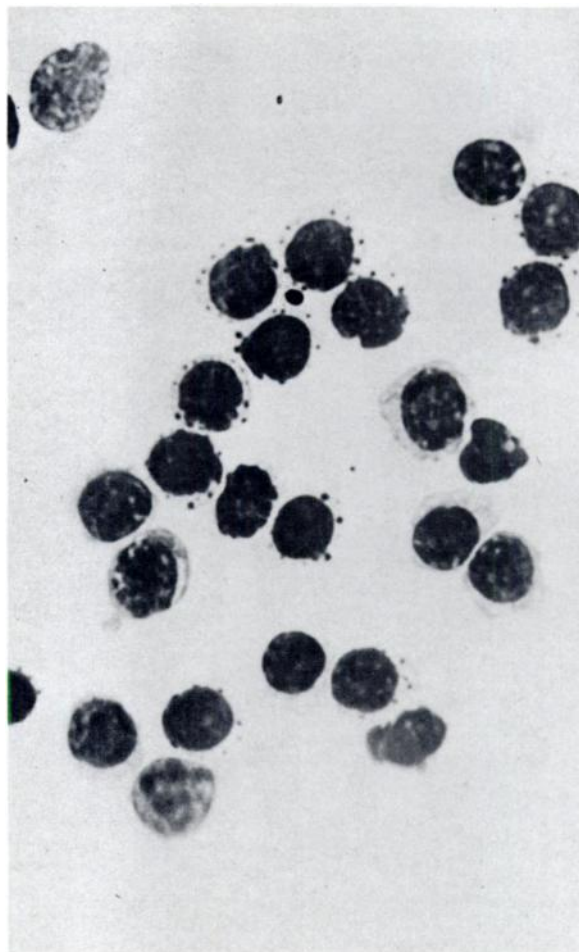


Fig. 3. Cells from fraction 7 of isokinetic gradient after separation of tonsillar cells as described in text. Lymphocytes with cytoplasmic inclusions were concentrated in this fraction. Wright stain. $\times 400$.

to lymphocytes.^{6,7} Inclusions in these cells stain with the alcian blue-safranin technique at pH 1.0. Fewer than 1% of cells in suspensions of tonsillar cells stained with this technique. No such cells were observed following sedimentation in fractions containing the modal population of LWCI. Similarly, in all patients, cells with histochemically demonstrable acid phosphatase or catalase were reduced in concentration (as compared with unseparated cells) or totally lacking in fractions from the gradient containing LWCI. In contrast, gradient fractions containing concentrated LWCI stained by Wright stain contained similarly concentrated cells with cytoplasmic inclusions with histochemically demonstrable succinic dehydrogenase. The correlation in the distribution of cells with these markers was striking (Fig. 2).

Because of the correspondence between these two markers, we suspected that the cytoplasmic inclusions that stained with Wright stain were mitochondria. From the same gradient, fractions with high and with low concentrations of LWCI by light microscopy (Figs. 3 and 4) were examined by electron microscopy (Figs. 5 and 6); fractions of concentrated LWCI contained many cells

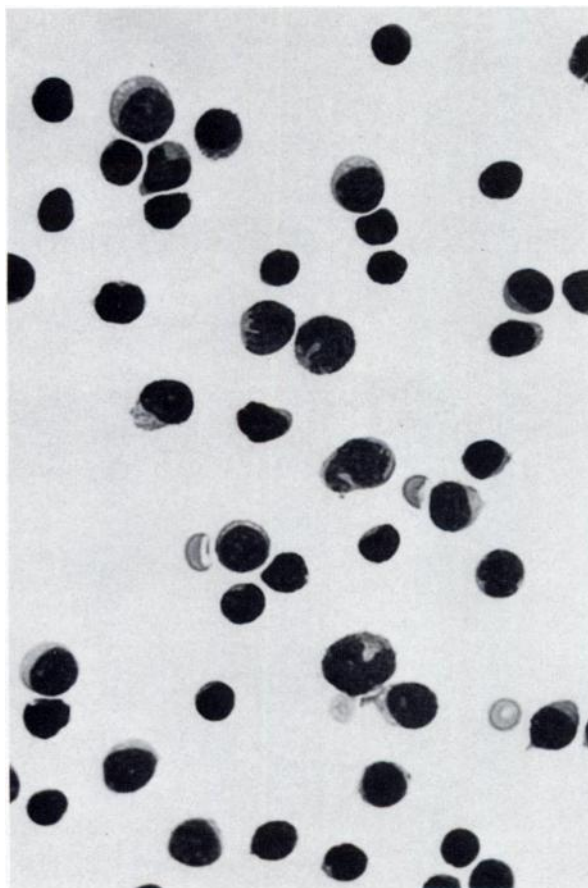


Fig. 4. Cells from fraction 9 of isokinetic gradient after separation of tonsillar cells as described in text. Modal population of all lymphocytes was found in fraction 9. Wright stain. $\times 400$.

with large mitochondria not found in fractions concentrated with respect to lymphocytes but depleted of LWCI.

DISCUSSION

We report a small subpopulation of human tonsillar lymphocytes that differed from other human tonsillar lymphocytes in that they contained cytoplasmic inclusions that stained blue with Wright stain. After separation of cells in an isokinetic gradient,⁴ these cells were found in the same fractions as lymphocytes with large mitochondria ultrastructurally. In addition, there was a precise correlation between the distribution of cells with these inclusions and cells with cytoplasmic inclusions with histochemically demonstrable succinic dehydrogenase. These data convinced us that the inclusions in these lymphocytes were large mitochondria. Neither large mitochondria nor inclusions with succinic dehydrogenase are found in cells in fractions with concentrated lymphocytes lacking inclusions stained by Wright stain. Interestingly, we found lymphocytes that we could not distinguish from LWCI among cells separated from human bone marrow and from human carcinoma; this work will be described in detail in a future report.



Fig. 5. Ultrastructural appearance of cells shown in Fig. 3. Note large number of prominent mitochondria in cytoplasm of these lymphocytes. $\times 9000$.

The presence in LWCI of inclusions (1) that stained with Wright stain and (2) with histochemically demonstrable succinic dehydrogenase proved to be a useful marker for LWCI; the absence of such inclusions in most lymphocytes raises an important question about the nature of these inclusions. If one is convinced by the data presented here that these inclusions are large mitochondria, one must wonder why such inclusions are not observed in the majority of lymphocytes. Are the mitochondria in LWCI only larger and more numerous than those in other lymphocytes, or are they chemically different from mitochondria of the other cells? It is our opinion that we saw the mitochondria by light microscopy in LWCI when stained with Wright stain or stained for succinic dehydrogenase because they were larger than most mitochondria in most cells; however, resolution of this problem would require ultrastructural, histochemical, and morphometric investigations that we did not perform.

There have been numerous reports of unusual cytoplasmic organelles and inclusions in lymphocytes. These include ribosome-lamella bodies in the lymphocytes of patients with several kinds of lymphoid neoplasms,¹³⁻¹⁵ tubuloreticular structures (thought by some to be viral) in lymphocytes of patients with lupus erythematosus,^{16,17} and tubular structures in lymphocytes from normal subjects¹⁸ and subjects with chronic lymphocytosis,¹⁹ lymphoproliferative dis-

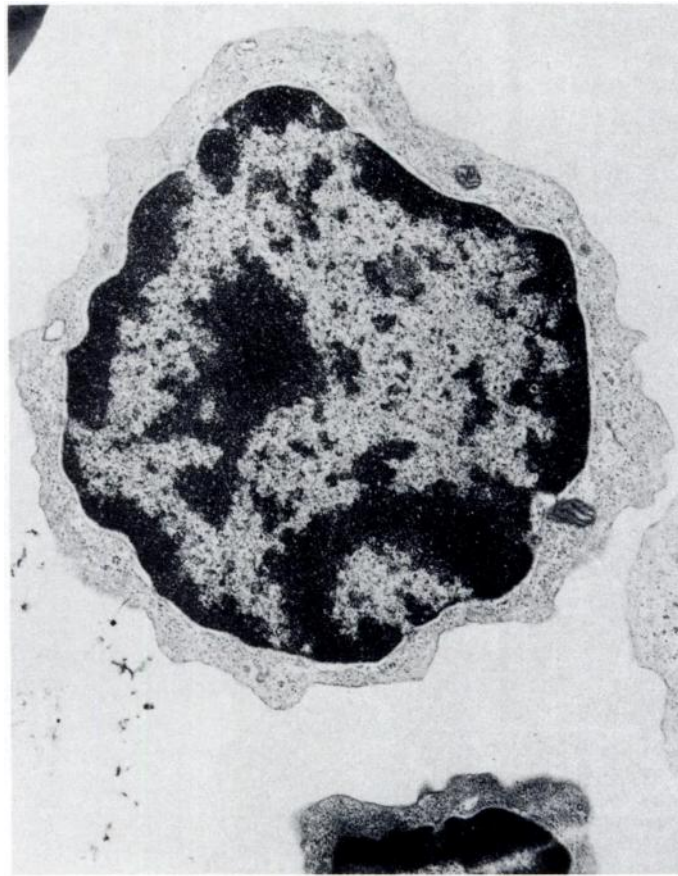


Fig. 6. Ultrastructural appearance of cells shown in Fig. 4. Cells that comprised modal population of tonsillar lymphocytes (fraction 9, ± 1 fraction) contained fewer, less prominent mitochondria than those in fraction 7 (Fig. 5). $\times 9000$.

orders,^{15,20} rheumatoid arthritis,²¹ and Chediak-Higashi syndrome.²² Tubular aggregates have also been described in cultured human lymphoblastoid cell lines²³ and in human lymphoid tumors after heterotransplantation.²⁴ Prior to our ultrastructural studies of tonsillar cells depleted of or enriched in LWCI, we wondered if LWCI might be related to the "globule leukocyte"²⁵⁻²⁷ or "epithelial lymphocyte"^{28,29} described in association with the epithelial lining of the alimentary canal in several species; however, the inclusions in gut-associated cells are clearly ultrastructurally distinct from the large mitochondria we observed in LWCI from human tonsils. The fact that these cells sedimented as a discrete subpopulation of lymphocytes suggests that they are different from other tonsillar lymphocytes; however, the precise role of these cells in the lymphoid system needs further elucidation.

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