Localization of Human T Lymphocytes in Tissue Sections by a Rosetting Technique

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A technique for the formation of sheep erythrocyte (E) rosettes in frozen human tissue sections is reported. The labile nature of the receptor for E rosettes on lymphocytes requires the use of controlled conditions for tissue processing and the reaction with indicator cells. The distribution of E rosettes in sections of normal human thymus, lymph nodes, tonsils, and spleens was comparable to that of the T marker-positive cells identified by immunofluorescence with the specific anti-human T cell serum. There was no overlap with areas positive for 19S EAC and 7S EA rosettes. Erythrocytes treated with a sulfhydryl reagent, 2-aminoethylisothiuronium bromide (AET), and with neuraminidase formed better rosettes in sections than did untreated erythrocytes. E rosettes in tissue sections can determine changes in the distribution of T cells in different lymphoproliferative and infiltrating disorders. (Am J Pathol 88:323–332, 1977)

T LYMPHOCYTES can be identified in the peripheral blood and cellular suspensions of lymphoid tissue by their ability to form rosettes with untreated sheep erythrocytes (E rosettes).[1,2] Little is known about the nature of the receptor on T cells that is responsible for this interaction. This receptor may be unstable, because E rosettes cannot be formed on cryostat sections or paraffin-embedded material.[3] Identification of mononuclear cells within tissues has been limited to cells which bear complement receptors and are thus capable of forming stable 19S EAC rosettes[4–6] and to cells with the receptors for the Fc portion of IgG which rosette with E coated with 7S anti-E serum.[6,7]

This communication describes a technique which is suitable for studies of the distribution of E rosette-forming lymphocytes in human tissues.

Materials and Methods

Preparation of Biopsy Material

Fresh, unfixed surgical specimens were obtained from the Pathology Laboratories of the Presbyterian Hospital, University Health Center of Pittsburgh, Pittsburgh, Pa. The biopsy specimens, including lymph nodes, spleen, and thymus, were cut into small pieces, wrapped in aluminum foil, and snap-frozen in liquid nitrogen. The tissues were

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stored in liquid nitrogen or at −85 C in a Revco freezer until frozen sections were made. Whenever possible, a portion of lymphoid tissue was used for the preparation of cellular suspensions. Thymus tissue was obtained from children of less than 4 years of age who underwent open heart surgery. Lymph nodes and spleen were obtained from patients having diagnostic biopsies and splenectomies, respectively. Normal lymph nodes and spleen from autopsies were also used.

Cell Suspensions

The tissue was teased apart, chopped into very small pieces in phosphate-buffered saline (PBS), pH 7.2, containing 0.1% EDTA. No wire mesh screens were used, for they tended to disrupt the cells. The cell suspension was layered over Ficoll-Hypaque and centrifuged as previously reported.[8] The interface cells were recovered and washed two times in medium 199 (Grand Island Biological Co.); the cell concentration was adjusted to 2 to 4 × 10^6/ml. Viability was determined by trypan blue dye exclusion and was generally higher than 90%.

Rosette Formation in Suspensions

E rosettes in suspensions were formed by the method of Jondal, Holm, and Wigzell [1] modified as described previously.[9] 19S EAC, 7S EA, and 19S EA rosettes were formed exactly as described by Ehlenberger and Nussenzweig.[10]

Tissue Sections

Tissue for cryostat sections was removed from storage and placed in Tissue-Tek II O.C.T. compound (Lab-Tek Products); 4- to 6-μ sections were cut with an Ames cryostat and mounted on clean gelatin-coated glass slides. Sections for immunofluorescent staining or for EAC and EA rosettes were allowed to air dry. Sections for E rosettes were *not* allowed to air dry and were processed immediately as described below.

Rosettes in Frozen Tissue Sections

19S EAC, 19S EA, and 7S EA rosettes were formed as described by Edelson *et al.*[7] The indicator cells were prepared according to Ehlenberger and Nussenzweig's procedure.[10] For E-rosette formation, glass slides containing 6-μ frozen sections were overlayed with 1% suspension of sheep erythrocytes in dextrose-gelatin-veronal-buffered saline with calcium and magnesium added: DGVBS+ buffer.[10] The overlaying had to be timed carefully so that the section would not air dry. If the E-cell suspension was applied to a wet section immediately after sectioning, the tissue did not stick to the slide. Therefore, a period of about 10 to 30 seconds, its length depending on temperature and humidity in the laboratory, had to lapse between sectioning and application of the indicator cells. The indicator cells were prepared fresh and kept at 37 C prior to their use. Sections layered with the indicator cells were kept in a humidity chamber for 30 to 45 minutes. The sections were then carefully washed three times with cold PBS, fixed with 3% glutaraldehyde solution in PBS for 30 minutes, washed, and stained with hematoxylin and eosin.

Staining of Frozen Tissue Sections

After air drying and fixation in 90% acetone for 2 minutes, the cryostat sections were washed in PBS for 10 minutes and stained with fluorescein-labeled antiimmunoglobulin sera (Meloy Laboratories, Springfield, Va.) or with anti-human T cell serum prepared in our laboratory as previously described.[9]

Results

The technique described for the formation of E rosettes in frozen tissue sections had to be performed under carefully controlled conditions. Since the receptor for E rosettes on T lymphocytes appears to be labile, optimal conditions for its stability in frozen tissue sections had to be defined. Thymus tissue was used for this purpose. Successful results depended on the following parameters: a) freezing and storage of the tissue, b) processing of the frozen sections, c) medium used for suspending of E cells, d) concentration of E cells, e) temperature of indicator cells, and f) time of incubation with E cells.

Snap freezing of tissues in liquid nitrogen is a preferable but not obligatory way of preparation for the human thymus. Tissue frozen in a −70 C Revco freezer for extended periods of time (several months) was as reactive as fresh, snap-frozen thymus. However, for other lymphoid tissues and patient material, freezing and storage in liquid nitrogen were necessary for good results.

Cryostat sections which were allowed to air dry gave negative results, suggesting the E receptor on lymphocytes was destroyed by drying.[3] On the other hand, application of the indicator cells to a freshly cut wet section resulted in washing off and a loss of the section or part of it. The exact number of seconds that may be allowed to obtain an adherent and still reactive section can only be approximated (30 seconds) because of variations in the temperature and humidity of any laboratory.

Different media, e.g., PBS, 199 (Gibco), glucose-veronal buffer (Cordis), and DGVBS+ buffer,[10] were tried for preparation of indicator cells. The DGVBS+ buffer was essential for E rosette formation in sections. One percent red cell suspension in DGVBS+ was better than 2, 3, or 5% suspensions. These higher concentrations of indicator cells allowed for nonspecific sticking of erythrocytes to sections and contributed to a disruption and loss of sections during washing.

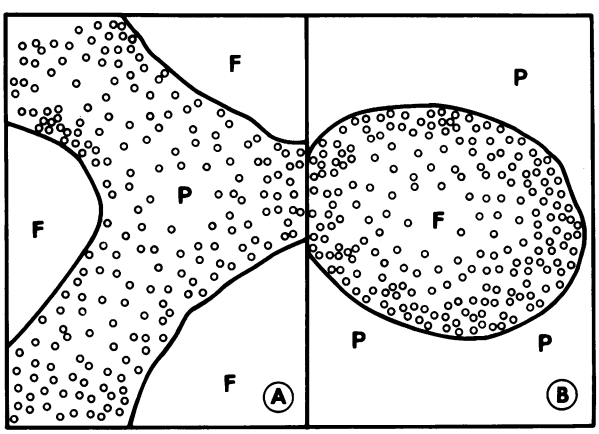
The indicator cells were applied to sections at various temperatures: 25 C, 25 C followed by cooling to 4 C, and 37 C. Warming of the indicator cells to 37 C in a water bath immediately prior to the application and allowing the reaction to take place at room temperature was found to be optimal. Finally, the time of incubation of the section with indicator cells was important. We found that an incubation longer than 45 minutes at room temperature was unsuitable, as it favored nonspecific sticking of erythrocytes and required excessive washing procedures which often disrupted the sectioned material.

It has been reported that the treatment of sheep red blood cells with

neuraminidase [11] or with a sulfhydryl reagent, 2-aminoethylisothiuronium bromide (AET),[12] enhances the reactivity of these cells with T lymphocytes without a loss in specificity of the interaction. We found that sheep erythrocytes treated with these reagents according to the published protocol[11,12] formed better rosettes in sections than did untreated erythrocytes. Reagent-treated erythrocytes were also compared with untreated erythrocytes for reactivity with T lymphocytes in cellular suspensions and normal peripheral blood. About a 2 to 10% increase in E rosettes was generally obtained with treated erythrocytes in suspensions, and the rosettes were larger and stronger. While thymus tissue in sections gave good E rosettes with untreated erythrocytes, AET or neuraminidase treatment was preferable for studies of E rosettes in normal lymph nodes, tonsils, spleen, and in pathologic tissues.

The technique was used to study the distribution of T lymphocytes in sections of normal human lymph nodes, tonsils, and spleens as well as some pathologic lymphoid tissues. Figure 1A shows E rosettes in frozen sections of the thymus. There was no nonspecific sticking of erythrocytes to thymus tissue (Figure 1B). The distribution of E rosettes in sections was compared to that detected with the specific anti-human T cell serum [9] by indirect immunofluorescence of fixed sections. As shown in Figures 1C and 2A, T lymphocytes in a normal human lymph node visualized with the antiserum and by means of E rosettes are limited to paracortical areas.

Sections of lymphoid tissues were always treated with 19S EAC, 19S EA, and 7S EA indicator cells for controls and to see if areas of overlapping with E rosettes could be identified. In normal human lymph nodes, spleen, and tonsils, no overlap with areas positive for 19S EAC (see



TEXT-FIGURE 1—Schematic drawings of a normal human lymph node sectioned and reacted with E and EAC indicator cells. **A**—The paracortex (*P*) is covered with E rosettes (*open circles*). The neighboring follicular areas (*F*) do not react with E. **B**—The follicular area is positive for EAC rosettes (*open circles*) and the paracortex is negative.

Figures 1C and D) or 7S EA rosettes were seen. However, in abnormal tissue, e.g., some lymphoma nodes, overlapping of E-positive and EAC-positive areas was observed, indicating that the abnormal lymphocytes may carry receptors for complement as well as a T marker. In addition, the distribution of E rosettes was shown to be exclusive of immunoglobulin-bearing lymphocytes in normal human tissues (Figures 1C and 2B).

Discussion

Surface markers have been successfully used to identify and differentiate mononuclear cell populations in peripheral blood and cellular suspensions of lymphoid tissues. EAC and EA rosetting techniques have been adapted to the use with sectioned material [6] and have yielded valuable data about the distribution of B lymphocytes and macrophages in normal and diseased lymphoid[13,14] as well as nonlymphoid tissues.[15,16] Immunofluorescence with specific anti-human immunoglobulin and anti-T cell sera [9] is applicable to studies of lymphocyte distribution in tissues. However, the scarcity of well-characterized anti-human T cell serum reduces the general applicability of this method of localizing T cells. An E-rosetting technique applicable to tissue sections was described by Silveira *et al.*[17] Unfortunately, the technique was not reproducible in this and other laboratories.[6] To identify mononuclear cells in tissue infiltrates, it was necessary to extract these cells from the tissue and then submit them to the same assays as circulating lymphocytes.[18]

The technique described here is simple, specific, rapid, and reproducible, provided care is taken to establish controlled conditions. The pretreatment of sheep red blood cells with AET increases the sensitivity of the E rosette formation in tissues where receptors for erythrocytes appear to be weaker or less dense than in the thymus or normal lymph nodes. While untreated erythrocytes bind strongly to the thymus tissue, their binding to T lymphocytes in pathologic tissue sections tends to be variable. We recommend that sections of normal human thymus and/or normal lymph nodes are always processed simultaneously with patient material as controls for E rosettes in tissue sections.

Several recent publications illustrated the application and importance of surface markers in identifying B cells and monocytes at local sites in a variety of disease states.[13-16] T cells were identified in human tissue sections by exclusion: mononuclear cells that were not B cells (IgM EAC positive) or monocytes (IgM EAC and IgG EA positive) were assumed to be T lymphocytes. In view of the functional heterogeneity of T-cell subpopulations,[19-21] as well as overlapping of markers on activated T lymphocytes,[22] the identification by exclusion appears inadequate. The

technique described here identifies those mononuclear cells that have a receptor for untreated E. To date only one laboratory has claimed to have succeeded in identifying T lymphocytes in sections of human tissues by the E rosetting technique.[17,23,24] However, neither we nor others have been able to reproduce and use Silveira's technique.

Recent data indicate that T lymphocytes are heterogeneous, comprising several functionally distinct subpopulations. Little is known about the tissue distribution of these T lymphocyte subpopulations. Certain T lymphocytes may play a pathologic role in disease, e. g., act as effector cells in certain lymphoproliferative states. Correlations between tissue sections and *in vitro* studies of infiltrating lymphocytes may provide information pertaining to the functional significance of T lymphocytes in disease processes. Further, the distribution of T lymphocytes in the lymphoid tissue and circulation changes in the course of a disease [9] (see Figure 3). The described rosetting technique offers a simple and reliable way of following these changes in relation to the progress and clinical manifestations of the disease.

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[*Illustrations follow*]

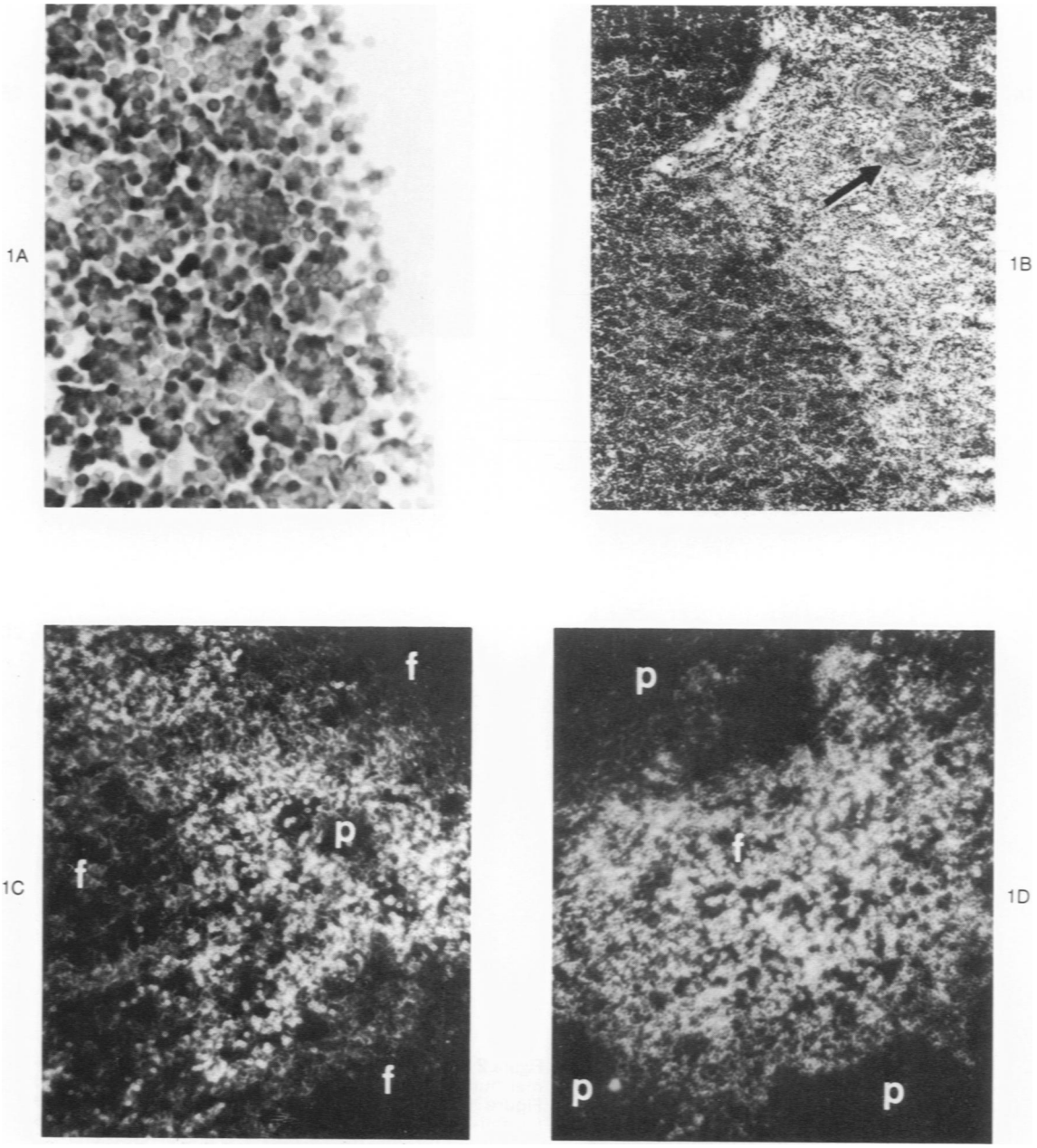
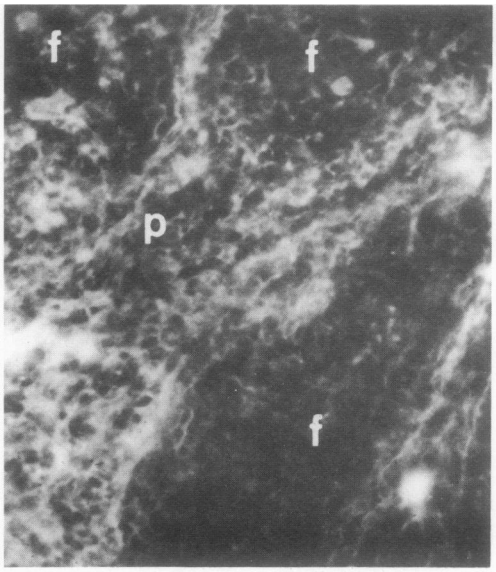


Figure 1A and B—E rosettes on frozen sections of a normal human thymus. Note numerous, distinctive rosettes at the edge of section in **A**. E rosettes are only seen in thymic cortex (dark areas in **B**). There are no rosettes in thymic medulla. The *arrow* indicates Hassall's corpuscle. H&E, **A**, × 770; **B**, × 125) **C and D**—Sections of a normal human lymph node corresponding to the drawing in Text-figure 1. E rosettes are seen only in paracortex (**C**) and EAC rosettes are seen only in follicles (**D**). *f* = follicle, *p* = paracortex. (Dark field, × 200)



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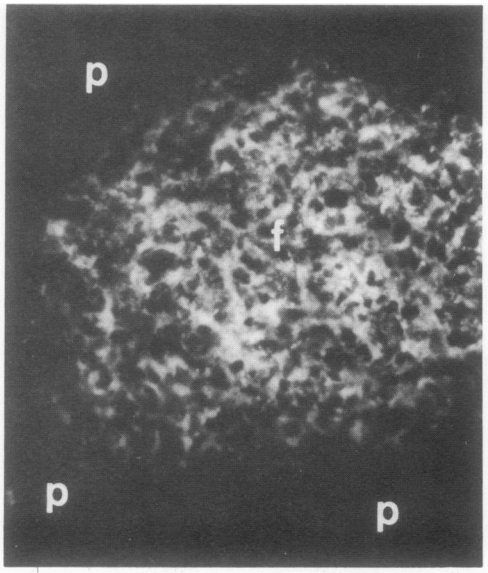

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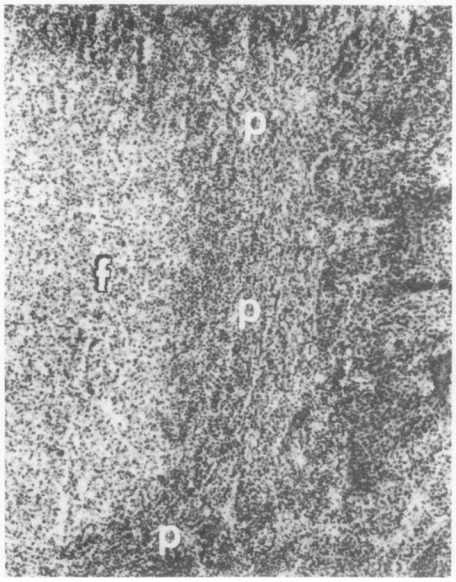

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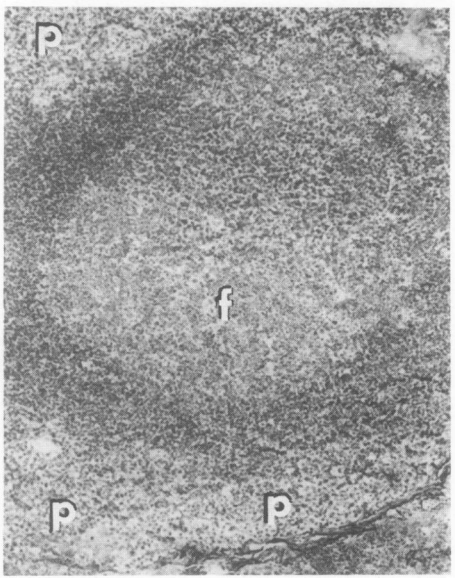

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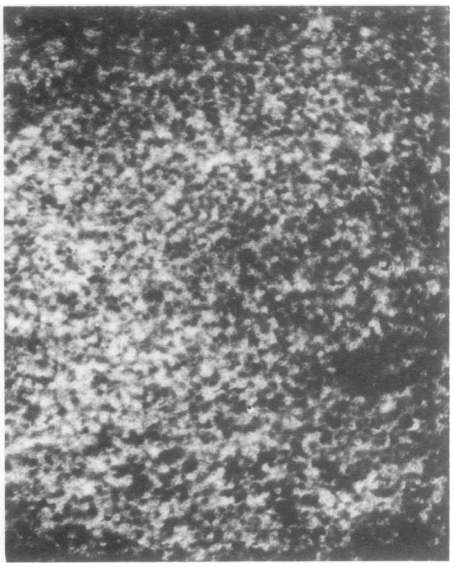

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Figure 2 A and B—Immunofluorescent staining of the normal human lymph node illustrated in Text-figure 1 and Figure 1C and D showing that paracortex (*P*) is positive for anti-human T cell serum (**A**), while the follicle (*f*) stains with antihuman IgM (**B**). (× 200) **C and D**—Sections of a human tonsil. E rosettes (**C**) are limited to the interfollicular regions (dark areas). Follicular areas are positive for EAC rosettes (**D**). Note the higher concentration of EAC rosettes in the outer follicular region and one central portion of the follicle. (H&E, × 125) **Figure 3**—E Rosettes are seen all throughout the section of a lymph node from a patient with Hodgkin's lymphoma (Dark field, × 200).