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Isolation and Characterization of Inflammatory Cells from the Human Periapical Granuloma

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Twelve histologically-confirmed periapical granulomas were evaluated by conventional immunologic rosette assays for the presence of T-lymphocytes and complement receptor-bearing lymphocytes. A technique for dispersing the granuloma cells into suspensions was adopted to facilitate performance of the assays which were not applicable to tissue sections. Differential cell counts by an acridine orange vital dye method disclosed that the cell suspensions contained 30% macrophages, 44% lymphocytes, 15% plasma cells, and 12% neutrophils. Complement receptor-bearing cells comprised 17.9%, and T cells comprised 34.5% of the unseparated inflammatory cells. This study provides the first direct evidence of a predominance of thymic-derived lymphocytes in the lymphocyte compartment of the periapical granuloma. Analysis of the data shows that cell-mediated immunity most likely plays a role in the pathogenesis of the periapical granuloma.

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Introduction.

The human periapical granuloma, an aggregate of inflammatory cells which accumulate at the end of a tooth root, has recently been shown to contain 47% macrophages, 32% lymphocytes, 13% plasma cells, and 8% neutrophils by differential analysis of 33 tissue biopsies.¹ While the macrophage is the predominant cell type and the "hallmark" of the granulomatous inflammation,^{2,3} the significant numbers of lymphocytes and plasma cells are highly suggestive of a role for the immune system in the evolution of the lesion. The presence of immunoglobulin-producing cells in the periapical granuloma has been documented,^{4,7} thereby implicating the humoral-immune system. Recently, Stern *et al.*⁸ found that 42% of the lymphocytes and plasma cells in these lesions displayed membrane or cytoplasmic immunoglobulins. The demonstration that the vast majority of the lesional lymphocytes are not antibody-producing indicates that most may be either T-lymphocyte members of the cellular-immune system, or undefined "null" lymphocytes. The presence of the thymic-derived nonimmunoglobulin-producing T-lymphocytes has been suspected,^{9,10} but the lack of generally accepted cytologic markers for T cell recognition in histologic sections has prevented positive identification. The usual methods of T cell delineation — by enzyme markers,¹¹ immunologically distinct membrane markers,¹²⁻¹⁷ and rosette assay^{18, 19} — while applicable to blood lymphocytes, are not suitable for tissue lymphocytes, because of the difficulties in applying the technique to paraffin-embedded sections.

To facilitate assessment of T-lymphocytes in the human periapical granuloma, a method was devised based on that of Mackler,²⁰ combining dicing tissue specimens with incubation in a solution of collagenase to produce dispersed

cell suspensions. Use of the cell dispersion method in the present study has permitted documentation of the presence of a significant T-lymphocyte compartment in human periapical granulomas.

Materials and methods.

Twelve granulomas obtained at surgery constituted the study series. The specimens were washed free of blood and immediately placed into 75 ml of sterile tissue culture medium[†] and transported to the laboratory. The specimens were bisected, and one half was fixed in 1% alcoholic formalin for histologic evaluation and diagnosis. The other half was weighed, was minced with a sharp single-edged razor, and then placed into a tube containing 2 ml of a collagenase mixture[‡] which was shaken in a water bath at 37°C until the fibrous part of the tissue was largely dissolved.

Enzyme digestion was halted by dilution of the collagenase with 3 ml of fresh tissue culture medium. The 5-ml mixture was gently dispersed with a Pasteur pipette. Fragments of undigested tissue were allowed to settle for five min before the cell suspension was carefully siphoned off with the pipette, was placed into a conical tube, and centrifuged at 150 x g for ten min. After the cell-free supernatant was discarded, the granuloma cells were resuspended in 5 ml of fresh tissue culture medium, and the procedure was repeated. The pellet was then resuspended in 1 ml of tissue culture medium.

Total inflammatory cell counts were made for each digested specimen after a 1:20 dilution of an aliquant with Turk's solution in a leukocyte-diluting pipette. Cells were counted in a Levy Ultra-Plane chamber[§] with a phase microscope. A separate aliquant was diluted 1:20 with 0.1% Trypan blue dye in saline for viability assessment. All cells that excluded the dye were judged viable. Those with cell membranes penetrated by the dye were rated non-viable.

Differential counts from dispersed granuloma cells were made by a vital dye method.²¹ An acridine orange-coated coverslip was placed over a drop of granuloma cells on a clean glass slide, and the cells were examined microscopically under ultraviolet illumination. Plasma cells appeared as cells with eccentric nuclei containing peripherally-clumped green-staining chromatin and diffuse green cytoplasm; lymphocytes as small round cells with homogenous green-staining nuclei and scant cytoplasm showing one or two orange vesicles; neutrophils as bi- or tri-lobed nucleated

[†]500 ml RPMI-1640 with glutamine (Grand Island Biologicals, Grand Island, NY), plus 5 ml 1 M HEPES buffer, 2.5 ml Gentamicin[®] (10 mg/ml), and 50 ml heat-inactivated fetal calf serum sterilized by filtration through a 0.22 μ millipore filter

[‡]The sterile tissue culture medium (RPMI-1640), plus 0.14 g CaCl₂ and 2.5 mg/ml collagenase (Worthington CLSPA collagenase, 400-500 units/mg dry wt, Worthington Diagnostics, Freehold, NJ) added just prior to use

[§]Becton, Dickinson, and Co., Parsippany, NJ

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cells with numerous bright orange-red cytoplasmic inclusions of dye; monocytes/macrophages as mononuclear cells with homogenous green-staining nuclei and abundant cytoplasm laced with large orange-red vesicles of pinocytosed dye.

The granuloma cells were then subjected to a rosette assay with sheep erythrocytes (SRBC). SRBC, diluted 1:2 in Alsever's solution, were washed three times with phosphate-buffered saline (PBS) and were resuspended in 10 ml RPMI-1640 tissue culture medium. The concentration was brought to $1.25-2.0 \times 10^8$ SRBC/ml for the assay. In the assay, 0.1 ml granuloma cells (adjusted to 10×10^6 mononuclear cells/ml where possible) was added to 0.1 ml fetal calf serum (adsorbed with SRBC's) in a centrifuge tube and was incubated in a shaking water bath at 37°C for 15-30 min. Subsequently, 0.2 ml washed SRBC was added to the tube, was mixed, and then centrifuged at 600 RPM (70 x g)

for five min. The gently pelleted cells were held in a refrigerator at 4°C for one h – whereupon the cells were carefully resuspended with a rocking motion, while the tube was kept in ice water. Following resuspension, 0.3 ml 1% gentian violet in PBS was added, and rosette counts were performed within three min using a hemacytometer. Lymphocytes which bound three or more SRBC's were considered positive (T) cells. A minimum of 200 cells was counted in each assay.

Complement receptor-bearing lymphocytes were identified by mixing an aliquant of the granuloma suspension with sheep red blood cells coated with 19S anti-sheep antibodies[†] and mouse complement (EAC reagent). For this test, 0.1 ml of the granuloma suspension (10×10^6 cells/ml) was added to 0.4 ml RPMI-1640 and 0.25 ml EAC reagent and was incubated in a shaking water bath for 30 min at 37°C. Cells which bound three or more SRBC's had receptors for the third component of complement and were scored as positive cells. At least 200 cells were counted. An illustrated summary of the methods used in the present study is shown in Fig. 1.

Results.

Microscopically, each specimen demonstrated the classic histologic characteristics of the periapical granuloma. Wet weights of the granuloma specimens ranged from 73.5 to 703.4 mg. The average dispersion time necessary to release cells into suspension in the collagenase incubation medium was 3½ h (range from three to five h). The mean number of mononuclear cells recovered per mg of tissue by enzyme dispersion was 21,087. The average total mononuclear cell recovery was 5.28×10^6 (range from 975,000 to 14,450,000). Viability of specimens tested within 20 h after surgical removal from the patient was 94% (90-98%) and dropped to 40% for the case tested 48 h after surgery.

Results of the differential cell counts by the acridine orange vital dye method of a minimum of 200 cells per slide from the biopsies yielded mean \pm standard deviation values of $30 \pm 9\%$ (18-41%) macrophages, $44 \pm 11\%$ (19-71%) lymphocytes, $15 \pm 6\%$ (8-27%) plasma cells, and $12 \pm 7\%$ (5-22%) neutrophils. Results of a statistical comparison to differential cell counts on tissue sections from a previous study⁸ are shown in Table 1.

Thymic-derived T-lymphocytes identified by spontaneous rosette formation with sheep erythrocytes (Fig. 2, top) and by acridine orange stain (Fig. 2, bottom) constituted

[†]Cordis Laboratory, Miami, FL

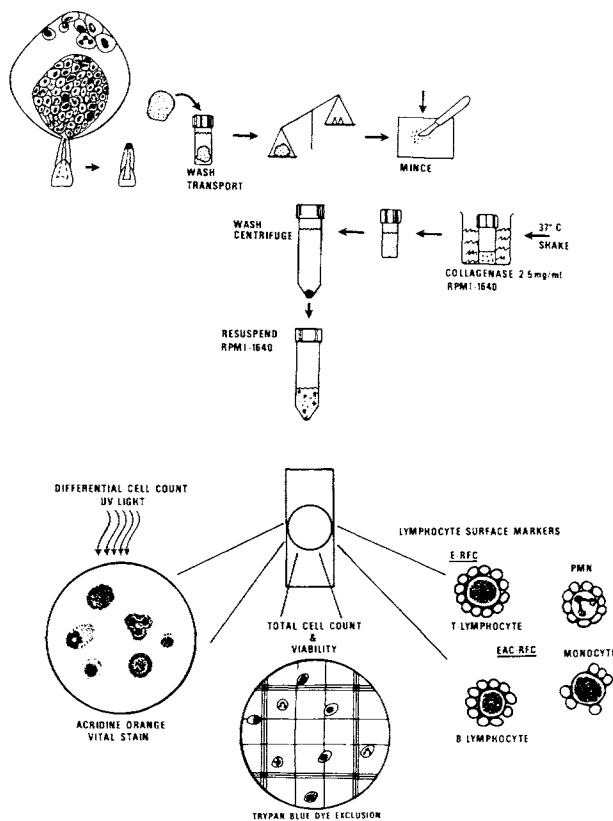


Fig. 1 – Enzymatic dispersion method.

TABLE 1
COMPARISON OF DIFFERENTIAL CELL COUNTS FROM TISSUE SECTIONS AND CELL SUSPENSIONS

| Cell Type | Tissue ¹ | | Suspension | | t | p | d |
|-------------|---------------------|------|-------------------|------|-------|------|-----|
| | Mean \pm s.d. | N | Mean \pm s.d. | N | | | |
| Macrophage | 46.51 \pm 11.47 | (33) | 30.36 \pm 8.90 | (11) | 4.027 | <.05 | 16% |
| Lymphocyte | 31.96 \pm 2.37 | (33) | 43.50 \pm 11.22 | (10) | 5.780 | <.05 | 12% |
| Plasma Cell | 12.94 \pm 7.41 | (33) | 14.90 \pm 5.95 | (10) | 0.272 | >.05 | 2% |
| Neutrophil | 8.11 \pm 6.69 | (33) | 11.78 \pm 6.94 | (9) | 1.616 | >.05 | 4% |

s.d. = standard deviation.

N = number of cases.

t = score from Student's test for differences between means.

p = probability.

d = absolute difference between mean percentages.

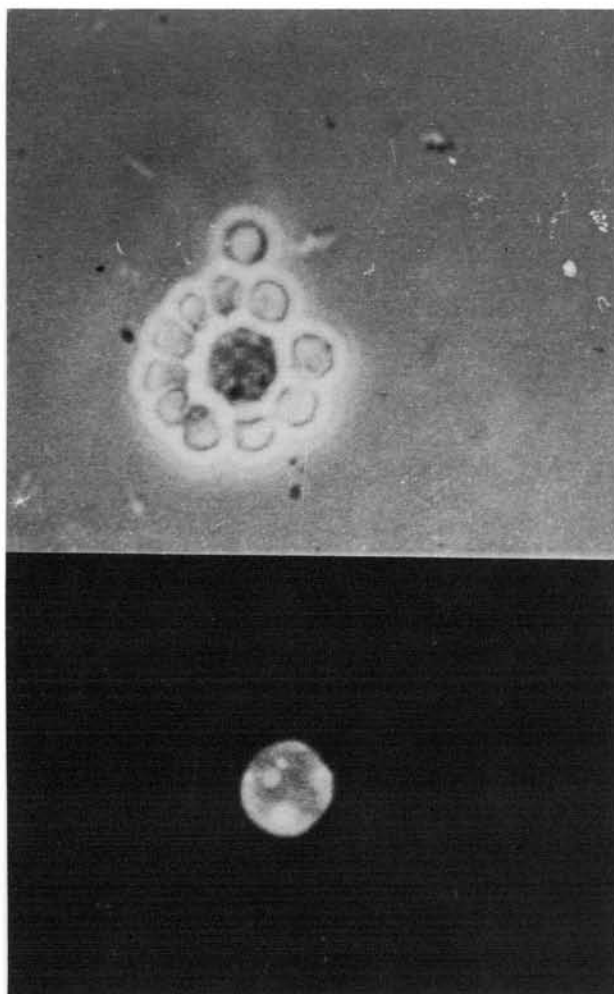


Fig. 2 - Top, phase contrast photograph of rosetted T-lymphocyte. Bottom, acridine orange/ultraviolet light photograph of same cell demonstrating lymphocyte morphology. (Original mag. x 400)

$34.48 \pm 15\%$ of the total mononuclear inflammatory cells (range 9-62%) and approximately 77% (34/44) of all the lymphocytes. Complement receptor-bearing cells (Fig. 3) comprised $18 \pm 10\%$ of the granuloma inflammatory cells (range 2-32%). Individual specimen values are listed in Table 2.



Fig. 3 - Phase contrast photograph of complement-receptor-bearing rosetted cells and non-rosetted mononuclear cells. (Original mag. x 400)

TABLE 2
GRANULOMA DISPERSION STUDIES OF 12 CASES

| Case | Weight Mg | Hold Time H | Digest Time H | Total Cell/mm ³ x 10 ⁶ | Viability % | E-RFC % | EAC-RFC % | M % | L % | P % | N % |
|-----------|--------------|----------------|------------------|---|----------------|------------|--------------|--------|--------|--------|--------|
| 1 | 237.10 | 18.50 | 4.0 | 14.450 | 97 | 37 | 21 | 18 | 42 | 27 | 13 |
| 2 | 103.45 | 20.00 | 3.5 | 5.100 | 90 | 56 | 11 | 31 | 48 | 15 | 5 |
| 3 | 281.30 | 18.00 | 4.0 | 5.900 | 95 | 38 | 20 | 35 | 42 | 20 | 3 |
| 4 | 161.60 | 19.00 | 4.5 | 3.175 | 98 | 48 | 8 | 43 | 19 | 16 | 22 |
| 5 | 703.40 | 17.75 | 4.0 | 5.500 | 94 | 9 | 23 | 18 | 42 | 19 | 22 |
| 6 | 261.50 | 16.50 | 3.5 | 5.250 | 95 | 23 | 20 | 29 | 46 | 14 | 11 |
| 7 | 73.50 | 18.00 | 3.0 | 2.750 | 91 | 31 | 32 | 41 | 39 | 8 | 14 |
| 8 | 341.75 | 0.00 | 3.5 | 7.000 | 90 | 33 | 30 | 34 | 51 | 8 | 11 |
| 9 | 467.00 | 16.50 | 2.5 | 1.880 | 95 | 23 | 28 | 38 | 42 | 12 | 5 |
| 10 | 88.00 | 4.50 | 4.0 | 1.750 | 97 | 62 | 15 | 27 | 64 | 10 | ND |
| 11 | 175.00 | 16.00 | 4.0 | 9.650 | 95 | 32 | 2 | 20 | ND | ND | ND |
| 12 | 112.00 | 48.00 | 3.0 | 0.975 | 40 | 22 | 5 | ND | ND | ND | ND |
| \bar{x} | 250.47 | 17.73 | 3.63 | 5.280 | 89.75 | 34.48 | 17.91 | 30.36 | 43.50 | 14.90 | 11.78 |
| s.d. | 184.32 | 11.36 | 0.57 | 3.800 | 15.89 | 15.06 | 9.83 | 8.90 | 11.22 | 5.95 | 6.94 |
| N | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 11 | 10 | 10 | 9 |

Hold time = time from biopsy to enzymatic dispersion in laboratory.

% = percentage rounded off to nearest integer.

RFC = rosette-forming cell.

ND = test not done.

\bar{x} = mean.

s.d. = standard deviation.

N = number of cases.

Discussion.

The human periapical granuloma is a relatively common lesion which can serve as a source of human inflammatory cells for the study of cell function in inflammation. The percentage distribution of macrophages, lymphocytes, plasma cells, and neutrophils and the proportion of antibody-producing cells in this lesion have been delineated.^{1,8} The present study was designed to elucidate the morphologic and immunologic characteristics of the lesional inflammatory cells, and to identify specifically thymic-derived lymphocytes by conventional assays.

All previous efforts to determine the immunologic nature of lymphocytes in periapical granulomas have focused on the identification of antibody-producing cells of the humoral-immune system.⁴⁻⁷ Only tangential data suggesting a contribution by the cell-mediated arm of the immune system have heretofore been presented.^{9,10} Documentation of a role for the cellular-immune system in the pathogenesis of the lesion is contingent upon demonstration of thymic lymphocytes in the periapical tissues. In this study, T-lymphocytes accounted for approximately 77% (34/44) of the lesional lymphocytes subjected to enzymatic dispersion and rosette assay analysis.

An enzymatic dispersion method adapted from previous work in this laboratory²⁰ was employed to produce granuloma cell suspensions. Human T-lymphocytes are conventionally identified by the ability to form spontaneous rosettes with sheep erythrocytes, but this assay is readily quantifiable only for cells in suspension. Withers²² has shown that collagenase has no significant effect on cell surface markers. Although protease, DNAase, and collagenase have been employed to produce cell suspensions from tissues,²³⁻²⁶ "purified" collagenase alone was used in this study to minimize nonspecific proteolytic enzyme damage to cell markers.

Results from the differential counts of inflammatory cells in suspension compared favorably with those obtained from analysis of tissue biopsies.⁸ Plasma cells and neutrophils were found in nearly the same percentage in both studies; monocytes and lymphocytes had a 16% and 12% difference, respectively. When suspension and tissue counts were compared, the absolute differences for each type of inflammatory cell were within the limits of biological variation. Furthermore, when lymphocytes and macrophages (monocytes) were combined into a single 'mononuclear cell' group, interstudy agreement was excellent (78% for tissue sections; 74% for cell suspensions).

When unseparated granuloma cells were reacted with complement-coated erythrocytes, 18% of the cells tested had receptors for the third component of complement. Although this assay is not specific for B lymphocytes,²⁷ the 18% value corresponds closely with the 19% immunoglobulin-positive cell figure obtained in our study of tissue sections.⁸

Conclusions.

The contribution of the cellular arm of the immune system to periapical inflammatory reactions has hitherto been studied only indirectly by lymphokine production and by blastogenesis assays in the peripheral blood of patients with periapical disease. Results have been contradictory. Eleazer *et al.*²⁸ failed to elicit a role for the cell-mediated system by peripheral blood blastogenesis assays in patients with periapical lesions, whereas Stabholz and McArthur¹⁰ documented lymphokine production by circulating lymphocytes in such patients. Peripheral blood assays may not be

reliable indicators, because the cells participating in the immune response are located within the lesion and do not circulate freely.

The presence of T cells in periapical lesions has not been previously demonstrated. The concurrence between tissue immunoglobulin staining and rosette assay findings was very close and indicates that T-lymphocytes are the predominant lymphocytic effector cells in periapical granulomas. The high percentage of functionally viable thymic-derived lymphocytes in the granulomas strongly suggests that the cell-mediated immune system plays a role in the pathogenesis of the periapical lesion.

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