Conjunctival-associated Lymphoid Tissue: Evidence for a Role in the Secretory Immune System

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A specialized lymphoid tissue in rabbit conjunctiva was studied by various histologic and immunologic techniques and compared with similar structures along other mucosal surfaces. The flattened conjunctival lymphoepithelium overlying the lymphoid follicles was devoid of goblet cells. This lack of goblet cells is characteristic of epithelium overlying similar lymphoid collections in gut and bronchus. The lymphoid follicles demonstrated neither intra- nor extracellular immunoglobulin, and the lymphocytes in these follicles were composed of B-cells and T-cells, when studied by various immunologic techniques. A high proportion of these lymphocytes showed surface immunoglobulin A (IgA), and a high proportion of IgA precursors were determined by pokeweed mitogen (PWM) stimulation in 4-day cultures. The morphologic and immunologic results are similar to those obtained from gut and bronchus, tissues known to disseminate lymphoid cells to other mucosal sites already committed to antigen and IgA isotype. It is speculated that conjunctival associated lymphoid tissue of rabbit is part of a generalized system of secretory immunity capable of sampling conjunctival applied antigen, and then disseminating cells committed to IgA antibody production to other mucosal sites. Invest Ophthalmol Vis Sci 25:181—187, 1984

Lacrimal gland has been demonstrated to contain a high proportion of both immunoglobulin A (IgA) plasma cells and adjacent acinar epithelial cells, that contain secretory component (SC), in humans and rabbits. These findings are consistent with observations in other mucosal-associated tissues and suggest that lacrimal gland constitutes part of a common mucosal immune system. The IgA plasma cells associated with various mucosal tissues originate from B-lymphocytes committed to IgA production found in gut-associated lymphoid tissue (GALT) and bronchus-associated lymphoid tissue (BALT) and migrate as committed B-lymphocytes to the various mucosal-associated tissues.

Specialized lymphoid structures located adjacent to small intestine epithelial cells (Peyer's patches [PP], a component of GALT) and to bronchus epithelial cells apparently encounter luminal antigen, initiating a sequence of events that results in a commitment to antigen-specific B-cells of the IgA isotype. Morphologic observations have suggested that a similarly specialized structure exists adjacent to conjunctival epithelium in the rabbit. This structure is composed of lymphoid follicles devoid of plasma cells with an overlying lymphoepithelium. The present experiments confirm several of the previously described morphologic characteristics of this conjunctival-associated lymphoid tissue (CALT) and add morphologic as well as functional characteristics of this tissue consistent with studies on PP and BALT.

Materials and Methods

Rabbits

New Zealand white outbred rabbits of both sexes, at least 16 weeks of age and weighing greater than 2.5 kg, were used in these studies. By hand light examination, all animals contained easily identifiable CALT in the inferior cul de sac of the conjunctiva and, in most instances, contained smaller patches of similar nodules in the superior conjunctival cul de sac. The rabbits were killed by an overdose of intravenous sodium pentobarbital.

Tissue Preparation and Histologic Technique

Tissues from six rabbits were immediately removed after sacrifice and placed into 10% neutral buffered formalin. Each tissue was sectioned with a razor blade. One-half of each tissue was embedded into paraffin, sectioned, and stained with hematoxylin and eosin, periodic acid-Schiff, and methyl green-pyronin. The remaining half of each tissue was prepared for...
cell lysis was accomplished by suspending the cell pellet were rewashed and then suspended in 5 ml of HBSS and 95%. HBSS with HEPES and glucose two times. Red blood cells were then centrifuged and resuspended at 4°C in buffer (pH 7.4) and 10 mM glucose. The suspended balanced salt solution (HBSS) with 10 mM HEPES and CALT were excised immediately after sacrifice in a 10-fold volume excess of HEPES-buffered amonium chloride, (nine parts 0.83% NH₄Cl:one part 0.2 M HEPES, pH 7.0), for 10 min at 24°C. The cells were rewashed and then suspended in 5 ml of HBSS at 4°C. The suspended cells were centrifuged over a Hypaque-Ficoll medium (5 parts 32.8%:12 parts 14%). Viable cell counts were obtained by trypan blue exclusion, and generally ranged between 85 and 95%.

Fluorescent Antibody Reagents and Fluorescent Microscopy

Fluorescein-conjugated goat anti-α, anti-γ, and anti-μ rabbit heavy chains were obtained from a commercial source (Cappel Laboratories, West Chester, PA) and were tested for specificity by immunoelectrophoresis and demonstration of accepted proportions of isotype-specific plasma cells in normal rabbit spleen, lymph node, and ileum. End-point dilutions were obtained for each reagent, and dilutions of reagents were selected for use that were two serial dilutions less than the end-point, usually 1:4 or 1:8. A fluoresceinated goat anti-human IgG serum (Cappel) was used as a fluoresceinated control serum.

Immunofluorescent stained tissue—CALT, PP, ileum, spleen, and lymph node—from each rabbit were examined separately with a Zeiss microscope equipped for epifluorescence. Each tissue was examined at multiple levels with adjacent serial sections. A xenon light source was used with filters specific for fluorescein.

Preparation of Single Cells

PP, spleen (SP), preauricular lymph nodes (PLN), and CALT were excised immediately after sacrifice from 22 normal rabbits, teased apart at 4°C in Hanks balanced salt solution (HBSS) with 10 mM HEPES buffer (pH 7.4) and 10 mM glucose. The suspended cells were then centrifuged and resuspended at 4°C in HBSS with HEPES and glucose two times. Red blood cell lysis was accomplished by suspending the cell pellet in a 10-fold volume excess of HEPES-buffered ammonium chloride, (nine parts 0.83% NH₄Cl:one part 0.2 M HEPES, pH 7.0), for 10 min at 24°C. The cells were rewashed and then suspended in 5 ml of HBSS at 4°C. The suspended cells were centrifuged over a Hypaque-Ficoll medium (5 parts 32.8%:12 parts 14%). Viable cell counts were obtained by trypan blue exclusion, and generally ranged between 85 and 95%.

Mitogens and Cell Culture Techniques

Concanavalin A (Con A; Difco Laboratories, Detroit, MI), and pokeweed mitogen (PWM; GIBCO Laboratories, Grand Island, NY) were reconstituted to manufacturer's specifications with sterile, deionized water to form stock solutions, and then stored at −20°C.

Stimulation of DNA synthesis in cells from various lymphoid tissues of six rabbits was evaluated in tissue culture plates with 96 flat-bottomed wells (Linbro, New Haven, CT). Cultures were prepared using 25 µl of RPMI-1640 medium (GIBCO) containing mitogen, 25 µl of 25% heat-inactivated normal rabbit serum in RPMI-1640 medium, and 25 µl RPMI-1640 medium containing cells. All cultures contained 2-mercaptoethanol at a final concentration of 5 × 10⁻⁵ M. The mitogen solutions were added to the wells at three times the final concentration. Dose response curves were obtained for each mitogen, and the optimal dose per well selected was 10 µg Con A and 10 µl of a 1:50 PWM solution. Ten to 12 wells were prepared per mitogen per dilution for SP, PP, and PLN, and four culture wells were prepared for CALT, the variability dependent upon cell recovery from each animal's tissues. The final concentrations of cells from each tissue was adjusted to equal the concentration of CALT cells for each experiment involving tissues from 6 rabbits. The cell concentrations were 5–8 × 10⁴ cells/well (2–3 × 10⁵ cells/ml). Control experiments consisted of an equal number of identically prepared wells, but treated with HBSS instead of mitogen. The culture plates were covered and incubated in a humidified atmosphere of 5% CO₂/95% air at 37°C.

At 84 hrs, 10 µl of tritiated thymidine (specific activity 24.7 Ci per mM; New England Nuclear, Boston, MA) in RPMI-1640 medium was added to each well. The cultures were terminated at 96 hrs and the cells were isolated from individual wells using a cell harvester (Hiller Co., Madison, WI). The individual filter papers representing individual wells were counted in a liquid scintillation counter (Beckman LS-7500; Beckman Instr., Irvine, CA), and the results were expressed in counts per min for mitogen and non-mitogen stimulated (control) cultures. The mean counts were calculated for each tissue of each animal, then a final mean was obtained for each tissue of the six rabbits.

The ability of PWM to effect differentiation of some rabbit lymphoid cells to plasma cells has been previously described.¹⁴ Mononuclear cells from either PLN, PP, or CALT were placed into microtiter wells at a final concentration of 1 × 10⁵ viable cells/well in 20% FCS with PWM at 7.5 µl/well (30 µl/ml). Tissues from four rabbits were examined separately. The culture plates were covered and incubated as described above. After 4 days, individual wells were pooled for each of the three tissues, washed with HBSS, and resuspended in 5% FCS at 4 × 10⁵ cells/ml.

Cytoplasmic immunofluorescence was used to examine cultures for plasma cells. The cells were deposited on glass slides using a cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, PA). The cells were fixed in acetone-methanol (1:2) for 10 min at...
These slides were then air dried and stored at −20°C until stained. Cytoplasmic immunoglobulin was detected using fluorescein-conjugated antisera by placing one drop onto the fixed cells and incubating for 30 min in a humid chamber. The slides were then washed twice in PBS for 10 min at room temperature. A cover slip was applied using mounting medium (Aquamount; Lerner Laboratories, New Haven, CT), and the slides were examined using the fluorescence microscope described above.

**Membrane Immunofluorescence of Cells from Normal Lymphoid Tissue**

Cells were suspended in fluorescein-conjugated goat anti-rabbit α, γ, or μ heavy chain specific reagents diluted with HBSS (1:4 or 1:8) for 12 min at 37°C. A fluorescein-conjugated goat anti-human IgG was used as a control serum. The cells were washed once and resuspended in HBSS supplemented with 5% FCS at 4 × 10^5 cells/ml. The cells were then deposited on glass slides using a cytocentrifuge and fixed in acetone-methanol (1:2) for 10 min at −30°C. A cover slip was applied, and the slides were examined using the fluorescent microscope described above. Cells were counted at ×160 magnification in eight separate fields for each of the three reagents. A fourth slide, stained with Wright-Giemsa stain, was similarly counted under bright field conditions to determine the total number of mononuclear cells per eight separate ×160 fields.

**Results**

**Histology**

The CALT tissue was composed of numerous lymphoid follicles immediately beneath and often encompassing the overlying conjunctival epithelium. The normal stratified conjunctival epithelial cells were often difficult to distinguish because of the intense infiltration of small lymphocytes. Goblet cells were not seen in the lymphoepithelium over the follicles with PAS stain, but could be seen in normal proportions immediately adjacent to this area (Fig. 1a). Occasional follicles had the appearance of secondary follicles with a central area of larger, less mature lymphocytes. No plasma cells were identified within any follicles. With methyl green-pyronin stain, it was possible to identify occasional plasma cells immediately adjacent to follicles.
Fluorescent Microscopy of CALT

Immunofluorescent examination of rabbit CALT showed only occasional plasma cells adjacent to the follicles, and both IgA and IgG plasma cells could be identified with a slightly greater proportion of IgA staining cells. Immediately adjacent to follicles and under the normal conjunctival epithelium, IgA plasma cells were prominent, with few cells staining for IgG. Only a rare IgM cell could be detected adjacent to follicles or epithelium. The follicle failed to show fluorescence with any of the antisera.

Cell Yields Obtained from CALT and PP

CALT tissue from both eyes of 22 separate rabbits yielded \(1.2 \times 10^5\) cells, with a viability range between 80 and 90%. When cell suspensions were isolated in HBSS without HEPES and glucose, the viability of CALT cells ranged from 50 to 80%. Wright-Giemsa-stained smears of the CALT cell suspensions showed greater than 90% small lymphocytes, with the remainder being macrophages and epithelial cells. Usually, five PP were isolated from each rabbit and, occasionally, as many as eight. Between \(3.0-8.0 \times 10^7\) lymphoid cells were isolated per animal, the variability dependent upon PP number and size per rabbit, with a viability of 85 to 95%. By Wright-Giemsa stain, greater than 95% of these cells were shown to be small lymphocytes and the remainder was a mixture of macrophages and other mononuclear cells.

Proliferative Responses to Mitogens

Dose-response curves were obtained for CALT, PP, SP, and PLN for each mitogen. Con A doses were 2, 10, and 50 \(\mu\)g/ml, and PWM doses were 10 \(\mu\)l of a 1:10, 1:50, and 1:150 dilution of the stock solution. Since so few cells were available from CALT, individual rabbits were used for each mitogen along with control wells. The maximum proliferation was consistently obtained in each tissue with 10 \(\mu\)g/ml of Con A and 1:50 dilution of PWM.

Considerable variation was found in the maximum proliferation between animals, ranging from very high responses for each tissue with each mitogen to low
responses. This variation is evident in the standard error of the mean (SEM) as shown in Table 1.

Con A responses were generally high but varied greatly from rabbit to rabbit and among the four tissues examined. PWM stimulation also varied among the individual rabbits, but the best responses were obtained with PP and PLN. SP and CALT consistently showed much lower responses than with Con A.

**PWM Induced Differentiation of Lymphoid Cells**

Cytocentrifuge preparations of cells were examined for cytoplasmic staining at the beginning and after 4 days of culture. In addition, controls consisted of cells cultured with HBSS in place of PWM for 4 days. Prior to culture, CALT and PP showed less than 0.35% cytoplasmic staining cells equally distributed among the three immunoglobulin isotypes. Spleen cells showed 1.0–1.5% cytoplasmic staining cells prior to stimulation, with a majority of IgG and IgM staining cells. Viable cell yields following four days of culture with PWM were 50–80% when compared with the cultures at Day 0. Control cultures (unstimulated) showed a recovery of less than 20%. The mean percentage of cytoplasmic staining cells in PWM-stimulated cultures after 4 days was 4.5% for PP, 18% for SP, and 12% for CALT. Four-day cultures not stimulated with PWM consistently showed fewer positively stained cells. The isotype distribution presented in Table 2 is a ratio of PWM stimulated to unstimulated cells from 4-day cultures performed separately on tissues from four rabbits. Both PP and CALT showed an increase (4.7-fold) in IgA staining cells as well as a slight increase in IgA staining cells in SP. The 4.7-fold increase in IgA cells in PP and CALT compared with SP was significant for CALT \( (P = 0.048) \) but less significant for PP \( (P = 0.06) \) by Student’s t-test. Large increases in IgM were also seen in SP and PP with a slight increase in CALT.

**Membrane Immunofluorescence**

CALT, PP, and SP cells from 12 rabbits were examined for surface immunofluorescence. PP consistently yielded the highest percentage of IgA staining cells, with fewer IgA B-cells in CALT, but significantly more than SP (Table 3).

**Discussion**

These present experiments examine a lymphoid structure observed consistently in rabbit conjunctiva (CALT)\(^{10,11} \) and provide additional morphologic and lymphocyte functional observations, consistent with findings on GALT and BALT tissue. In the present studies, the lymphoepithelium overlying CALT was found devoid of goblet cells, and the CALT follicles contained no intra- or extracellular immunoglobulin. Additionally, CALT tissue contained both T- and B-cells, with a large proportion of IgA-committed B-cells. Finally, these experiments demonstrated a proportion of B-cells in CALT capable of differentiation to IgA plasma cells after mitogen stimulation, similar to previous studies on PP\(^{12}\) and confirmed in the present studies.

The response of rabbit lymphocytes to Con A and PWM have shown that both mitogens stimulate T-cells; however, PWM acted on only a sub-population of T-cells in addition to stimulating some B-cells.\(^{15}\) The present experiments demonstrated a high response of all four lymphoid tissues with Con A and consistently lower responses with PWM. Similar observations have been seen in most rabbit lymphoid tissues by other investigators, and the decreased PWM responses were shown by them to represent a combination of responses from both B-cells and a sub-population of T-cells.\(^{15}\) Therefore, the response of CALT tissue to Con A and PWM (Table 1) indicates the presence of T-cells, although the PWM-sensitive component is less than in PP. Other studies have demonstrated thymus-specific antigens on a population of rabbit PP and BALT lymphocytes,\(^{16}\) which is similar to observations in other species.\(^2\) In the present studies using mitogens, rabbit

**Table 1. Comparison of responses to mitogens of normal rabbit tissues in four-day cultures**

<table>
<thead>
<tr>
<th></th>
<th>Con A</th>
<th>PWM</th>
<th>Control</th>
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<tbody>
<tr>
<td>CALT</td>
<td>92,000</td>
<td>15,500</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td>(23,500)</td>
<td>(6,800)</td>
<td>(150)</td>
</tr>
<tr>
<td>PP</td>
<td>140,000</td>
<td>73,400</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td>(46,000)</td>
<td>(17,100)</td>
<td>(450)</td>
</tr>
<tr>
<td>PLN</td>
<td>145,000</td>
<td>109,400</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>(77,000)</td>
<td>(39,600)</td>
<td>(70)</td>
</tr>
<tr>
<td>SPL</td>
<td>87,000</td>
<td>19,900</td>
<td>1,500</td>
</tr>
<tr>
<td></td>
<td>(31,300)</td>
<td>(8,600)</td>
<td>(332)</td>
</tr>
</tbody>
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* CPM, mean of responses for six experiments, 10–12 wells/experiment.
† SEM.
Four wells/experiment for CALT (for details, see Materials and Methods).

**Table 2. Differentiation of lymphoid cells after four days of culture (ratio of PWM stimulated to nonstimulated)**

<table>
<thead>
<tr>
<th></th>
<th>SP</th>
<th>PP</th>
<th>CALT</th>
</tr>
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<tbody>
<tr>
<td>IgA</td>
<td>1.2†</td>
<td>4.7</td>
<td>4.7</td>
</tr>
<tr>
<td>IgG</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IgM</td>
<td>4.4</td>
<td>6.9</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* Mean or ratio of four separate experiments (four rabbits).
† Ratio of cytoplasmic staining cells from PWM stimulated to nonstimulated.
CALT has been shown to contain a component of T-cells. PP and BALT have been shown to be an enriched population of IgA precursor lymphocytes. Membrane Ig studies on these tissues have demonstrated a higher proportion of cells with membrane IgA in BALT (41%) and PP (38%) as opposed to SP (17%). The present experiments agree with these observations (Table 3) and also demonstrate a high proportion of IgA B-cells in CALT. A more forceful demonstration of the potential for CALT lymphocytes to provide IgA precursors was obtained by 4-day cultures with PWM (Table 2). CALT and PP, but not SP cells, responded with a 4.7-fold increase in IgA plasma cells. In a similar series of experiments that employed the cell sorter in addition to PWM stimulation, a B-cell fraction from PP containing surface IgA was shown to be the direct precursor of IgA plasma cells. The experiments presented here, then, demonstrate that CALT is also a source of IgA precursors similar to PP and BALT.

It has been suggested that all mucosal surfaces are interrelated to some degree. Migration of antigen-committed IgA B-cells from the surface, where antigen encounter occurred to other surfaces, can proceed independently from prior antigen exposure at the second mucosal surface. Although antigen may play some role in the homing of the committed IgA B-cell, it is certainly not absolutely necessary. The mechanism is best understood for GALT, where a specialized epithelium covering the PP dome has been described. The PP dome epithelium is flattened with surface microvilli and contains no goblet cells. This specialized epithelium can transfer luminal antigen by pinocytosis into the lymphoid portion of the PP, and then cells committed to antigen and IgA isotype can migrate to other mucosal surfaces. Recently, antigen-specific secretory IgA (sIgA) has been demonstrated in tears, suggesting that ocular adnexal structures participate in this system of mucosal immunity, at least in their ability to accept IgA-committed B-cells.

Whether CALT can likewise sample antigens and then disseminate cells committed to antigen and IgA isotype to other mucosal sites has yet to be established. One experiment, however, suggests this possibility.

The right corneas of rabbits were immunized with heat-inactivated Herpes Simplex Virus, Type I (HSV). Neutralizing antibody of the IgA isotype was subsequently demonstrated in the tears of both eyes, although at much higher concentrations in the immunized right eyes. One interpretation of this HSV experiment is that HSV-committed IgA B-cells migrated from the right to left ocular adnexa.

Previous morphologic studies demonstrated that rabbit CALT contained specialized epithelial cells, identical to PP dome cells, as well as a lack of plasma cells in CALT, similar to GALT and BALT. The present experiments demonstrate further similarities among CALT, GALT, and BALT. The evidence suggests that CALT could function as a site of antigen absorption, followed by dissemination of B-cells to other mucosal surfaces where they can differentiate to antigen-specific IgA plasma cells.

Key words: Peyer’s Patch, conjunctival-associated lymphoid tissue (CALT), mitogen, immunoglobulin A, slgA, plasma cells

References

11. Axelrod AJ and Chandler JW: Morphologic characteristics of conjunctival lymphoid tissue in the rabbit. In Proceedings of

Table 3. Percentages of B-cell surface isotypes in tissues from normal adult rabbits

<table>
<thead>
<tr>
<th></th>
<th>IgA</th>
<th>IgG</th>
<th>IgM</th>
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<tbody>
<tr>
<td>CALT</td>
<td>28* (2.3)</td>
<td>18 (3.1)</td>
<td>55 (3.8)</td>
</tr>
<tr>
<td>PP</td>
<td>43 (2.6)</td>
<td>19 (2.9)</td>
<td>39 (1.9)</td>
</tr>
<tr>
<td>SPL</td>
<td>17 (1.0)</td>
<td>24 (3.5)</td>
<td>59 (3.0)</td>
</tr>
</tbody>
</table>

* Calculated as percentage of total IgA + IgG + IgM surface staining cells. SEM, N = 12.