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Identification of B- and T-lymphocytes in viral conjunctivitis using alpha naphthyl acetate esterase (ANAE) stain

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Abstract
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Degree Type
Thesis

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Identification of B- and T-lymphocytes

in Viral Conjunctivitis

using Alpha Naphthyl Acetate Esterase (ANAE) Stain

A thesis presented to the faculty of Pacific University in partial fulfillment of the requirements towards the degree of Doctorate of Optometry. March, 1981

Jeffrey B. Cooper
Jacob J. Krieg

Advisors: Diane P. Yolton, Ph.D.
Kevin D. Cooper, M.D.
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Forest Grove, OR

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Diane P. Yolton, Ph.D.

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United States Navy

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Researchers:
Jeffrey B. Cooper
Jacob J. Krieg

Advisors:
Diane P. Yolton, Ph.D.
Kevin D. Cooper, M.D.

Grade
ABSTRACT

This study utilizes a recently developed cytochemical staining tech­
nique to identify B- and T-lymphocytes in epithelial samples of virus
infected conjunctivae. The alpha naphthyl acetate esterase (ANAE) stain
has been used to differentiate lymphocyte subpopulations in tonsil, spleen,
lymph nodes and other tissues.

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and symptoms of the disease. The results indicated that the stain is
useful in conjunctival epithelial samples, and that B- and T-lymphocytes
were simultaneously present in about equal numbers in our patients. This
data confirms that of Belfort and Mendes who indentified both cell types
in inflamed conjunctiva by sheep erythrocyte rosetting techniques. The
ANAE stain is more easily performed, requires inexpensive substrates,
and uses common lab equipment as opposed to rosetting; and therefore
deserves strong consideration in future studies.
INTRODUCTION

The immunological response involves two types of lymphocytes, the B-lymphocytes and T-lymphocytes. The lymphocytes are responsible for the production of humoral antibodies, and the T-lymphocytes are responsible for the cell mediated response. Both B- and T-lymphocytes are originally derived from stem cells in the bone marrow and then differentiate into the thymus dependent (T-cells) and the thymus independent (B-cells). There is also another lymphocyte, the null cells, which belong to neither group. The majority of circulating lymphocytes are either B- or T-cells, with T-cells representing 60-70% of the total. Allansmith has shown that lymphocytes are present in biopsy samples of normal conjunctival epithelia, but they are found 20 times more concentrated in the deep substantia propria. This concurs with the relatively rare finding of lymphocytes in scrapings of normal conjunctiva, where only the epithelium is assayed. However, numerous studies have shown increased numbers of lymphocytes in scrapings and biopsies of the epithelium in inflamed eyes. Kimura and Thygeson reported that there are large numbers of lymphocytes found in scrapings from epidemic and herpetic dermatocconjunctivitis, acute follicular conjunctivitis, and conjunctivitis of other viral origins. Other authors state the lymphocytes are characteristic of acute viral infections and other chronic conjunctival infections. In these studies, the lymphocytes were not classified into T- or B-cells. Classifying the lymphocytes as B- or T-cells in these infections would be useful in order to determine whether the body's immunological response to mucosal surface viral infections is primarily B-cell, humoral mediated; or T-cell, cell mediated.
Belfort and Mendes used sheep erythrocyte rosetting assay to study biopsy specimens from patients with follicular conjunctivitis. They found B-cells clustered at the center of developed follicles with fewer T-cells in the peripheral areas. Rosetting, cytotoxicity, and immunofluorescence are the three main methods of lymphocyte differentiating. They all require large numbers of cells, sophisticated equipment, and complex preparation procedure. Staining procedures that do not suffer from these handicaps and that are currently used for conjunctival samples are Giemsa and Wrights stains. These stains can identify and differentiate lymphocytes from other leukocytes, but provide no differentiation among lymphocytes.

Recently, a stain differentiating B- and T-lymphocytes through alpha naphthyl acetate esterase activity (ANAE stain) has been successfully used to classify lymphocytes in thymus and tonsillar cell suspensions, leukemic blast cells, peripheral blood and tissue samples, and cultured lymphoid cell populations. The ANAE stain identifies T-lymphocytes through a characteristic marking pattern of the esterase activity. The stain has not been previously applied to conjunctival specimens and may be useful in characterizing immune responses in conjunctival tissue. Thus, in this study, T-lymphocytes were identified and quantified in samples of conjunctival epithelia from patients with signs and symptoms of viral conjunctivitis using the ANAE stain.
MATERIAL & METHODS

Subject Selection

Patients from local family practices who presented signs and symptoms of viral conjunctivitis were selected by physicians for the scraping procedure. A check list was used by the physicians to determine the inclusion of patients in the study. The initial selection process was based upon the following signs: current or recent upper respiratory tract infections, swollen preauricular or cervical lymph nodes, and injected or inflamed eyes. The final diagnosis of viral conjunctivitis was made from the data included above, plus the presence of a watery discharge, conjunctival follicles, a negative bacterial culture, and absence of any other overt causes.

Sampling Technique

The conjunctival scrapings were done in the standardized manner. Topical proparacaine (1 drop 0.5%) was placed in the lower conjunctival sac. This was followed by scraping the epithelium of the inferior tarsal conjunctiva with a sterile platinum spatula. The collected material was then spread as evenly as possible on a glass slide.

Staining Technique

The staining technique used was a modification of Knowles. The prepared slide was fixed at 4 degrees centigrade for 10 minutes in freshly prepared Formalin solution on the same day as the sample was taken. The Formalin solution contained 1 gm CaCl₂, 10 ml 37% Formalin, 90 ml H₂O and then stained for 19-24 hours in freshly prepared ANAE stain. The stain was prepared as follows:

1) Solution A:
   1 gram Para Rosanilin Base
   20 ml H₂O
5 ml conc. HCl
Warm gently, cool, filter. May be stored in the dark
at 4 degrees centigrade indefinitely.

2) Solution B
Prepare fresh each time.
0.048 gram Na Nitrite
1.2 ml H₂O

3) Add 1.2 ml of solution A to 1.2 ml of solution B and shake
until amber.

4) Add 2.4 ml of above to 40 ml of 0.067 M PO₄ buffer, pH 5.0.

5) Disolve 10 mg Alpha naphthyl acid in 10 ml acetone.

6) Add (5) into (4) and pour into Koplan jar.

After prescribed staining time, the slide was washed with H₂O for
15 minutes in 1% Methyl Green (1 gm Methyl Green, 100 ml 0.1 M acetate
buffer pH 4.2, filtered). The slide was then rinsed briefly with D H₂O
and final preparation was done by dipping the slide 10 times each in
sequence in 95% Ethanol, 100% Ethanol, and Xylene. The slide was dried
and covered for permanent use with a No. 1 or 2-13mm² glass coverslip and
Eupret fixative.

Microscopic Examination Of Slides

Slides were examined under 400X magnification for the presence of
ANAE activity and nuclear configuration indentified by the Methyl Green
counterstain. Positive ANAE activity was determined by a globular red
appearance in the cytoplasm. Cells were counted as ANAE (+) and ANAE (-)
lymphocytes, monocytes, and Polymorphonucleocytes (PMN).

Results

Five patients who presented signs and symptoms of viral conjunctivitis
were selected by local family practitioners for conjunctival sampling. Signs and symptoms varied from individual to individual with only upper respiratory infection and conjunctival injection being common in all five subjects. (Table I)

Of the five patients displaying signs and symptoms of viral conjunctivitis, three had conjunctival samples which showed both types of lymphocytes along with polymorphonuclear leukocytes and two had samples which showed only a few scattered epithelial cells with no leukocytes present. Of the three samples displaying ANAE activity, two of the samples (Samples 1 and 2) show similar numbers of cells bearing ANAE positive activity (52% and 46%) compared to ANAE negative activity (48% and 54%). One sample (Sample 4) showed 90% ANAE positive cells. Conjunctival samples taken from control subjects who displayed no signs or symptoms of ocular disease showed neither leukocytes nor lymphocytes (Table II).
<table>
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<th>REPORTED SIGNS &amp; SYMPTOMS OF CONJUNCTIVITIS</th>
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* Upper respiratory infection
** Control
**TABLE II**

*Cells Present in Epithelial Samples*

<table>
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<tr>
<th>Sample Number</th>
<th>PMN</th>
<th>(-) ANAE</th>
<th>(+) ANAE</th>
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* Control

** Very diffuse smear, too few cells of any kind to allow Counting.
DISCUSSION

The results indicate that there is often a mixed inflammatory reaction in presumed viral conjunctivitis involving PMN's, B- and T-lymphocytes, and monocytes. The quality of the samplings and smears was irregular, and in 2 of 5 slides (Samples 3 and 5), very few cells appeared on the prepared slide. In the slides that were successfully prepared (Samples 1, 2, 4, 6, 7, 8), it was found that the epithelial cells of the conjunctivitis had marked ANAE activity. This observation is confirmed by prior observation of epidermal skin biopsies stained with ANAE, in which dividing layer and mature differentiated squamous cells are ANAE positive (K. Cooper - unpublished observation). Because of this intrinsic ANAE activity in epithelial cells, the inflammatory cells could only be well visualized where they had been separated from epithelial cell clusters.

Slide 1 showed the highest percentage of ANAE+ lymphocytes (52%), and this patient had classical viral conjunctivitis signs and symptoms along with a history of chronic lid margin inflammation. The lymphocytic response consisting of mixed T- and B-lymphocytes could be in response to both the chronic and acute inflammation. Slide 2 showed a large number of polymorphonuclear leukocytes (PMN), usually indicative of a bacterial infection. The possibility of a bacterial infection in this patient was substantiated by the follow-up report that the condition had cleared up within 48 hours of institution of antibiotic therapy. The lymphocyte populations were again mixed, with both ANAE positive and negative lymphocytes present; the ANAE(-) cells predominated slightly. Slide 4 showed mainly ANAE(-) lymphocytes, and the patient had a history of prior conjunctivitis along with acute inflammation. The control slides
(Samples 6, 7, 8) showed only epithelial cells; no inflammatory cells were present. Slides 3 and 5 were not usable; they either contained very little cellular material from the sample or else the material was accidentally washed off during the procedure.

Lymphocytes showing ANAE(+) activity are interpreted to be mature "helper" T-cells. Grossi, et al. found that 70-80% of T-lymphocytes identified by sheep erythrocyte rosettes were ANAE(+). T-cell sub-populations bearing surface receptors for IgM (Tm cells) were found to be 95% ANAE(+). These Tm cells comprise a majority of circulatory T-cells and have been identified as "helper" T-cells in that they assist immunoglobulin synthesis by B-cells. Tm cells lose their ANAE(+) reaction within 4 days when stimulated by appropriate antigen. The reciprocal subpopulation of T-cells, Tg, bear receptors for Fe of IgG, suppress helper Tm cells and are ANAE(-). The lymphocytes, then, that are ANAE(-) can include several subpopulations: a vast majority (greater than 75% in all studies) of B cells, null lymphocytes, Tg "suppressor" lymphocytes, and previously activated Tm cells.

The lymphocyte population found in our samples agree with the data of Belfort and Mendes in that both T- and B-lymphocytes were present in samples from patients with presumed viral conjunctivitis. Our procedure samples only the very surface layers of epithelium, and differences in cell populations could be expected with different sampling procedures. The sampling technique and the ANAE stain are much less invasive and involve no expensive reagents or equipment that other techniques for classifying T- and B-lymphocytes require. However, variability in the quality of the sample makes quantification difficult and, thus the sampling technique needs to be precise and constant. The epithelial cells
possess ANAE activity that can overshadow that of the lymphocytes, so the Methyl Green counterstain time had to be increased to provide sufficient contrast. Although the preparation of the stain is straightforward, extended staining time (19-24 hours), fresh preparation of reagents, and strict pH requirements were required for reliable results. Once meeting all the requirements in sampling and reagent preparation, the stain is a valuable tool for identifying conjunctival lymphocytes infiltrations from surface samples.
REFERENCES


5. Cooper, K.D. Personal Correspondence.


REFERENCES (Cont.)