

Developmental Changes in Conjunctiva-Associated Lymphoid Tissue of the Rabbit

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PURPOSE. Organized conjunctiva-associated lymphoid tissue (O-CALT) is constantly exposed to environmental antigens and plays a central role in the common mucosal immune system. This study was undertaken to investigate whether O-CALT in rabbit lymphoid tissue, as in other lymphoid tissue in other mammals, changes with age.

METHODS. Fluorescence stereomicroscopy was used to measure the number and size of conjunctival follicles stained with propidium iodide in rabbits ranging in age from 2 days to 57 months. To assess the function of M cells, an antigen-sampling cell type found in the follicle-associated epithelium, transcytosis of fluorescent latex beads was evaluated with confocal microscopy.

RESULTS. O-CALT was not present in rabbits at birth, but appeared less than 24 hours after eyes opened at approximately day 11. The number of follicles increased with age until adolescence (2–4 months), when the number stabilized through early adulthood (17–20 months). In aged rabbits (47–57 months), there was a dramatic decline in the number of follicles. This disappearance was most pronounced in the superior conjunctiva. Average follicle diameter increased with age, except in the superior conjunctiva of aged rabbits, where the few remaining follicles were generally smaller. The uptake of latex beads showed that M-cell function was similar in all age groups.

CONCLUSIONS. Age-related changes in rabbit O-CALT are similar to those that have been reported for the human conjunctiva. Preferential uptake of latex beads by follicle-associated epithelium indicates that the presence and function of M cells are not affected by aging. The lower level of O-CALT in young and elderly animals, however, would be expected to decrease their ocular mucosal immune responses. (*Invest Ophthalmol Vis Sci.* 2008;49:644–649) DOI:10.1167/iovs.07-0856

The structural and functional characteristics of mucosa-associated lymphoid tissue (MALT) are similar in mucosae as diverse as the conjunctiva, gut, bronchus, and nasopharynx. Organized MALT consists of lymphoid follicles that are covered by a layer of specialized epithelium. This follicle-associated epithelium functions as the afferent arm of the mucosal immune system. M cells, a morphologically and functionally unique epithelial cell type within the follicle-associated epithelium, transport environmental antigens into the follicle to initiate the host immune response. Diffuse MALT consists of the

interfollicular IgA-secreting plasma cells and cytotoxic T cells, dispersed throughout the lamina propria, that are responsible for the efferent arm of mucosal immunity.

How changes in MALT contribute to the higher prevalence of infectious disease in mucosal tissues of neonates and the elderly is poorly understood. Mucosal immunity, like systemic immunity, undergoes a developmentally regulated reorganization that results in shifts in lymphocyte population profiles and altered responses to novel antigens during the course of an animal's lifespan.^{1,2} Whether variations in the size and number of lymphoid follicles contribute to an altered immune response in the neonate and the elderly appears to depend on the age, location, and species.^{1,3–8}

Age-related changes in O-CALT are known to occur in humans,⁹ but the lack of an animal model that undergoes similar changes has precluded the ability to test experimentally how changes in O-CALT may affect the ocular immune response to pathogens or allergens. Rabbits have been shown to contain O-CALT similar to humans^{10–14} and are commonly used as models of human ocular diseases. The objectives of the present study were (1) to quantify the conjunctival lymphoid follicles present in rabbits from birth to senescence, (2) to assess the relationship between lymphoid follicle size and age, and (3) to determine by latex bead uptake the functionality of M cells at different ages.

METHODS

Animals

Pasteurella free New Zealand White Rabbits (Harlan, Indianapolis, IN) were used for all studies. Rabbits were divided into groups based on age (Table 1). Neonatal rabbits (<4 weeks of age) were obtained by purchasing pregnant rabbits from the vendor. Only rabbits of normal health status with no obvious ocular abnormalities were used. All experimental procedures in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the University of Missouri Animal Care and Use Committee guidelines.

Latex Beads and Fluorescent Labels

Green fluorescent 0.5- μ m polystyrene latex beads (Fluoresbrite plain YG microspheres; Polysciences, Warrington, PA) were suspended in phosphate-buffered saline (PBS; 138 mM NaCl, 2.67 mM KCl, 1.47 mM KH₂PO₄, and 8.06 mM Na₂HPO₄ [pH 7.4]).

Surgical Procedure

The rabbits were sedated with an intramuscular injection of ketamine HCl (35 mg/kg) and xylazine (5 mg/kg). Green fluorescent latex beads (20 μ L of 1.0 \times 10⁹ beads/mL) were instilled at 0, 10, 20, and 30 minutes into the right inferior conjunctival sac of at least three rabbits aged between 12 days and 20 months. Experiments using old (47–57 months) rabbits were performed earlier in the study, before the inclusion of latex beads in the protocol. At 50 minutes after the initial instillation of latex beads, the rabbits were euthanatized via intravenous (adults) or intracardiac (neonates) injection (Beuthanasia-D; Schering-Plough Animal Health Corp., Kenilworth, NJ), and both eyes were exenterated. The lenses were removed, and the eyes were cut along the medial and lateral canthi. The superior and inferior conjunc-

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TABLE 1. Age and Gender of Rabbits

Group	Age Range	Females	Males	Total Eyes
Aged	47-57 months	3	0	6
Mature	17-20 months	3	0	6
Adolescent	2-4 months	5	7	24
Weanlings	29-37 days	3	6	18
Suckling	18 days	1	4	10
Neonates after eye opening at day 11	12 days	2	3	10
Neonates before eye opening	2-8 days	5	7	24

tivae were stretched out by pinning the corners of the eyelids onto a flat rectangle of dental wax. Mounted eyes were rinsed vigorously in PBS six times for 15 seconds each, to remove any extraneous beads and then were immediately placed in 2% PF (2% freshly depolymerized paraformaldehyde) in HEPES wash buffer (HWB: 70 mM NaCl, 30 mM HEPES, and 2 mM CaCl₂; pH 7.4). The time from the initial instillation of latex beads until the tissue was placed into fixative averaged 67.7 ± 4.4 minutes.

Processing for Fluorescence Microscopy

After 2 hours in fixative, the eyes were rinsed three times for 10 minutes each in HWB. After the extraneous tissue was removed, the superior and inferior eyelids were incubated overnight in fresh 2% PF+7.5 μM propidium iodide (Invitrogen-Molecular Probes, Eugene, OR) in HWB. Specimens were rinsed in HWB+50 mM glycine three times for 10 minutes each to block any remaining reactive groups on the aldehyde fixative. Fluorescently labeled wholemounts were viewed with a stereomicroscope under epifluorescence illumination, to allow enumeration and measurement of conjunctival follicles. Randomly selected individual follicles and control regions from the palpebral, bulbar, and fornix regions were dissected and either processed for confocal microscopy or embedded. To prepare cross sections of tissues for light microscopic examination, follicles were dehydrated with an ethanol series and embedded in methacrylate resin.¹⁵

Tissues selected for analysis by confocal microscopy were blocked for 1 hour in 1% bovine serum albumin (BSA), incubated overnight in 10 μg/mL of the fucose-binding lectin *Ulex europaeus* agglutinin (UEA)-I conjugated to biotin (Vector Laboratories; Burlingame, CA), and counterstained with 1 μg/mL streptavidin Alexa 647 (Invitrogen-Molecular Probes) for 4 hours. UEA-I stains the surface of an unidentified subpopulation of cells in the follicle-associated epithelium and intracellular mucin in goblet cells of control regions and was therefore used to identify the apical surface in both the follicle and control tissues.

Confocal Microscopy

To determine whether functional M cells capable of transcytosis were present over conjunctival follicles, we used confocal microscopy to evaluate the uptake of fluorescent latex beads. Five follicles were randomly selected from the inferior conjunctiva of three animals from each of the 12-day-old, 18-day-old, 4- to 5-week-old, 2- to 4-month-old, and 17- to 20-month-old age groups. For imaging, tissues were mounted between two coverslips in the presence of the antifade agent 1% 1,4-diazabicyclo [2,2,2]octane (Aldrich Chemical, Milwaukee, WI) in 200 mM Tris buffer (pH 8.6). Images were collected from the center of the follicles with a laser scanning confocal microscope (LSM 510; Carl Zeiss Microimaging, Inc., Thornwood, NY). After the apical surface of the follicle-associated epithelium or control regions by were established by UEA-I staining, the distribution of green fluorescent latex beads was visualized by collecting a z-stack of 80 optical sections measuring 225 × 225 μm at a 0.45-μm step interval for a total depth of 35.55 μm. A corresponding stack of images was obtained with a 633-nm laser line excitation of UEA-I labeling to ensure that only beads within the follicle-associated epithelium or directly under it were counted. This second stack was collected with a z-step of 5 μm,

because the level of resolution required in the z-axis was much less than with the 0.5-μm beads. All images were captured at a 2000 × 2000 resolution with a 40× plan apochromat water-immersion objective (NA = 1.2), using bidirectional scanning at a speed of 8 and a frame averaging of 2. The 30-mW, 488-nm and 5-mW, 633-nm lasers were used at 4% and 50% power, respectively, with a gain of 750 and an offset of -0.4 for both lasers. The green and red channel pinholes were set at 1 Airy unit with optical section thicknesses of <0.9 and 1.2 μm, respectively.

Evaluation of Follicle Size

Digital photomicrographs of superior and inferior conjunctival wholemounts acquired by fluorescent stereomicroscopy were evaluated from three animals from each age group (12 days to 57 months). The small (<200 μm), medium (200-300 μm), and large (>300 μm) lymphoid follicles were quantified (Photoshop 9.0; Adobe Systems, Inc., San Jose, CA). Follicles less than 100 μm were not found in animals older than 18 days of age and were only rarely observed in 12- and 18-day-old rabbits.

Statistics

Data summarizing the number of follicles in each age group are reported as the mean ± SD. An unpaired *t*-test was used to compare the number of follicles in each age group with that found in mature adult (17-20 months) animals. A paired *t*-test was used to compare the number of follicles in either the left versus right eyes of the same animal or the superior versus inferior conjunctivae of the same eye. A one-way analysis of variance (ANOVA) was used to compare the mean uptake between different age groups. A paired *t*-test was used to compare bead uptake in follicle-associated epithelial regions with the corresponding uptake in a nonfollicular palpebral control region from the same eye. All statistics were calculated with commercial software (Minitab 15; Minitab, Inc., State College, PA).

RESULTS

Quantification of Follicles

Wholemounts of propidium iodide-stained conjunctiva were evaluated by fluorescent stereomicroscopy (Fig. 1). Follicles were observed throughout the fornix and palpebral regions of the rabbit conjunctiva but rarely in the bulbar region. Propidium iodide gives an intense red fluorescent emission when bound to nucleic acids and, therefore, brightly labels the nuclei of all cells. Conjunctival follicles are densely packed collections of lymphocytes with little cytoplasm and stain more brightly than surrounding tissues, which simplifies their visualization and enumeration. Furthermore, follicles were also identifiable based on their characteristic dome shape, elevation above the epithelial surface, and presence of encircling blood vessels. When representative samples of these brightly stained, putative follicular regions were examined in 0.5-μm semithick cross sections, they were inevitably found to be follicles (Fig. 2). This staining approach is routinely used in our laboratory to identify follicles in the rabbit conjunctiva, and its reliability has

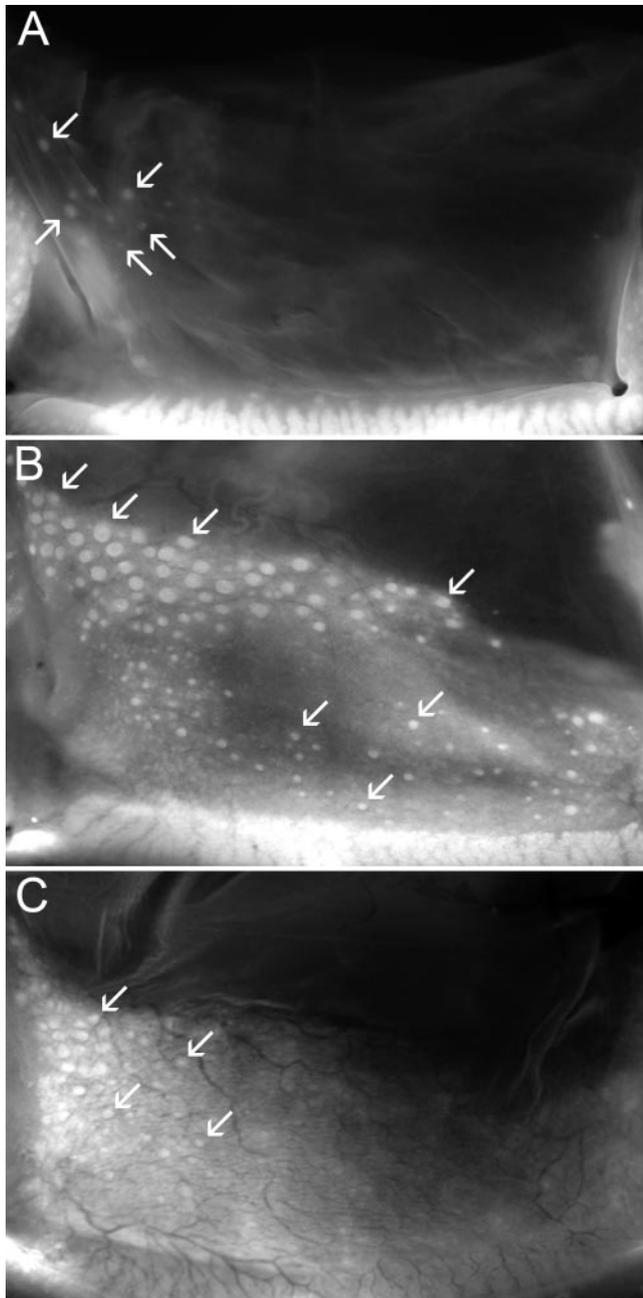


FIGURE 1. Inferior conjunctivae from left eyes of rabbits at (A) 12 days, (B) 2.3 months, and (C) 47 months oriented with the bulbar region at the top and the lid margin at the base of the image. Whole-mounts were incubated in the nuclear stain propidium iodide overnight and examined by fluorescent stereomicroscopy. Each white dome (arrows) represents one lymphoid follicle. In neonatal (A) and aged rabbits (C), follicles are primarily observed in the nasal fornix region. In young adults (B), fornical follicles are larger and more tightly packed nasally. Follicles decrease in size and are more spatially separated toward the temporal and palpebral regions (B). It is rare to find follicles in the bulbar region at any age.

been confirmed in a larger number of animals than reported in the present study.

After observation of a varying number of follicles in rabbits being used in other studies in our laboratory, a systematic examination of the number of follicles and the presence of macromolecule-transporting M cells in the follicle-associated epithelium as a function of animal age was conducted (Fig. 3).

The average number of follicles per eye was highest (138 ± 12.3) in mature rabbits between 17 and 20 months of age. The number of follicles/eye in prepubescent rabbits (2–4 months), however, was 98.7% of this level and was not significantly different. Sexual maturity in New Zealand White rabbits occurs at approximately 5 months in females and at 6 to 7 months in males.¹⁶ No follicles were detected in newborn animals before their eyes opened but they appeared rapidly after the eyes opened on day 11. On day 12, within 24 hours of eyes opening, neonates had 23.9% of the number of follicles per eye present in mature rabbits. The number of follicles increased to 56.4% of mature levels by day 18 and 68.7% after 29 to 37 days. In aged rabbits (47–57 months), there was a dramatic decrease in the number of follicles per eye to 32.6% of that found in mature adults.

There was no significant difference between the number of follicles in the superior and inferior conjunctival halves in rabbits that were 12 ($P = 0.077$) and 18 ($P = 0.876$) days of age. In both weanlings (29–37 days) and adolescents (2–4 months), the superior conjunctiva had, on average, 83.6% of the number of follicles found in the inferior half ($P = 0.032$ and 0.010, respectively). In mature adults (17–20 months), the superior conjunctiva had, on average, 62.9% of the number found in the inferior half ($P < 0.001$). This difference was even more pronounced in the aged rabbits (47–57 months) where the superior half had only 5.9% of the number present in the inferior half ($P < 0.001$). The 67.4% decline in follicles per eye present in the aged rabbits was a result of significant decreases in both the superior (4.7% of the number found in 17- to 20-month-old animals; $P < 0.001$) and inferior (50.4%; $P < 0.001$) halves.

The average difference between the number of follicles in the left and right eyes at all ages (12 days to 57 months) was $7.23\% \pm 5.4\%$ (range, 4.0%–17.7%) and was never significantly different within any single age group (range, $P = 0.074$ –0.547). The number of follicles per eye, in relation to gender, was compared in the two age groups in which there were at least three animals of each sex. Four-week-old females were found to average 30.0% more follicles per eye than males ($P = 0.018$). This difference was lost by adolescence (2–4 months) with the females having 1% fewer follicles than males ($P = 0.603$). It was not practical to look at the gender difference in older animals (≥ 17 months), because commercial suppliers rarely keep the males beyond 4 months of age.

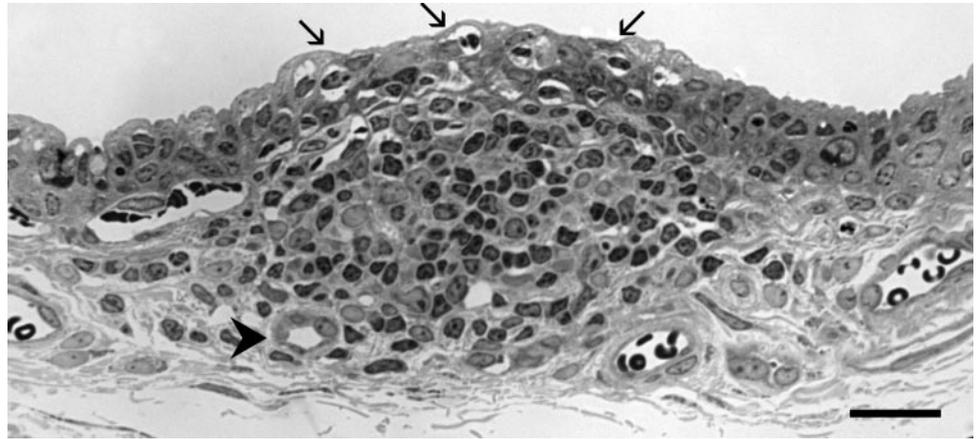
Follicle Diameter as a Function of Age

The average follicle diameter increased with age except in the superior conjunctiva of aged rabbits (Fig. 4). In animals 37 days of age or younger, more than 80% of the inferior follicles and more than 90% of superior follicles were less than $200 \mu\text{m}$ in diameter, and none were more than $300 \mu\text{m}$ wide. By 2 to 4 months, the diameter of 41.2% of inferior follicles and 33.9% of superior follicles was greater than $200 \mu\text{m}$. This trend continued in 17- to 20-month-old animals where 51.5% of the inferior follicles and 40.2% of the superior follicles were greater than $200 \mu\text{m}$. In aged rabbits, 62.2% of the inferior follicles remained over $200 \mu\text{m}$ in diameter, but in the superior conjunctiva, only 8.3% of the few remaining follicles were larger than $200 \mu\text{m}$, and none was greater than $300 \mu\text{m}$.

Evaluation of M-cell Function with Latex Beads

Although fluorescent stereomicroscopy was sufficient to see the preferential association of latex beads with the follicle regions (Fig. 5A), it could not resolve whether the beads were bound to the surface or had been translocated across the epithelium. Demonstration of transcytosis is essential, since that is the hallmark of functional M cells in the follicle-associ-

FIGURE 2. Light microscopic section of a conjunctival follicle of an 18-day-old rabbit stained with toluidine blue. Typical characteristics of CALT are present: the follicle-associated epithelium lacks goblet cells and overlays a dense collection of lymphocytes. Invaginations of the basolateral membranes of M cells (arrows) create pockets that contain lymphocytes. High endothelial venules (arrowhead), the site of lymphocyte migration, are present in the follicle region. Bar, 20 μm .



ated epithelium. To address this, confocal microscopy was used to quantify bead uptake in five randomly chosen follicles from the inferior conjunctiva of three rabbits in each of the age groups from 12 days to 20 months. Follicles in all age groups showed selective uptake (Fig. 6). The number of latex beads per individual follicle varied widely in a single eye with a few follicles having no beads and others as many as 1146 beads/ mm^2 after 50 minutes. For a single eye, the average number of beads taken up per square millimeter of follicle-associated epithelium ranged from 188 to 395 (average, 298.7 ± 86.5). There was no statistically significant difference in uptake at different ages ($P = 0.39$, ANOVA). In contrast, most z-stacks acquired from nonfollicular palpebral control regions had no beads and, on average, bead uptake in individual eyes ranged from 1.3 to 14.5/ mm^2 (average, 7.4 ± 6.1) for the different age groups.

DISCUSSION

This study demonstrates the presence of conjunctival lymphoid follicles in rabbits as early as 12 days of age. The number of follicles reached their maximum shortly before puberty, remained steady until at least 20 months, and then declined dramatically in aged rabbits. The demonstration that latex beads were actively transcytosed at all ages indicates antigen-sampling M cells were present at all stages of development once follicles appeared.

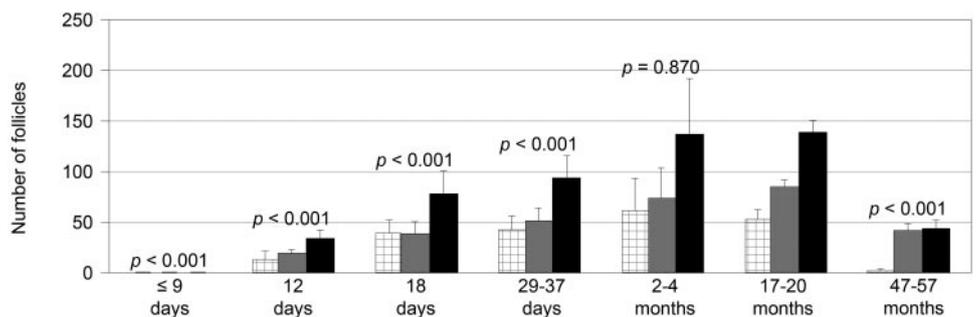
The use of propidium iodide to visualize and quantify lymphoid follicles proved to be a sensitive and reproducible method. Unlike earlier follicle staining techniques involving histochemical nuclear dyes,^{9,11,17,18} this fluorescent probe does not require special fixation or clearing techniques and the tissue can, therefore, be used downstream in immunofluorescence and electron microscopy studies. An earlier analysis of O-CALT in rabbits, using methylene blue staining, did not detect follicles less than 200 μm in diameter.¹¹ Propidium

iodide staining in the present study, on the other hand, revealed that most of the follicles at most ages were less than 200 μm ; histology of cross sections confirmed that this staining was due to the presence of follicles. The inability of methylene blue staining to detect smaller follicles would account for the failure of an earlier study to find follicles in 2- and 4-week-old rabbits and the lower number they observed in adults.¹¹

O-CALT is present in rabbits, guinea pigs, ferrets, pigs, cats, cows, dogs, sheep, nonhuman primates, and humans.^{9,11-14,17-21} The absence of CALT in mice and rats¹² precludes the use of these common laboratory species as models to study the role of lymphoid follicles and the specialized follicle-associated epithelium in ocular immunology and disease. Rabbits and guinea pigs not only have conjunctival lymphoid follicles, but have been shown to have M cells in the overlying follicle-associated epithelium that can transcytose lectins, latex beads, and bacteria. This makes them useful model systems for experimental testing of the role of O-CALT in the human conjunctiva.^{13,14,19,22}

Age-related decreases in mucosal immunity have been identified and postulated to be an underlying cause of the high rates of morbidity and mortality associated with mucosal diseases in the elderly.²³ In the intestinal tract, mucosal immunosenescence has been linked to reduced homing of IgA plasma cells to the lamina propria and impaired production of regulatory T cells.^{23,24} The failure to see age-related changes in the number of the clusters of intestinal lymphoid follicles known as Peyer's patches led some to speculate that follicle loss was not an important factor in intestinal mucosal immunosenescence in mice and rats.^{1,5} These studies, however, failed to measure age-related changes in the number of isolated lymphoid follicles that also play a major role in the intestinal mucosal immunity; the mouse small intestine, for example, has 100 to 200 isolated lymphoid follicles in addition to the 6 to 12 Peyer's patch clusters of follicles.^{25,26} Furthermore, age-related decreases in MALT have been found in numerous human tissues.

FIGURE 3. A comparison of the average number of follicles per eye by age group and location (batched bars: superior conjunctiva; gray bars: inferior conjunctiva; black bars: total eye). After rapid growth in the early stages of development, the number of follicles declined dramatically in aged animals. The probability above each group refers to the comparison with the total number of follicles per eye in mature rabbits at 17 to 20 months of age.



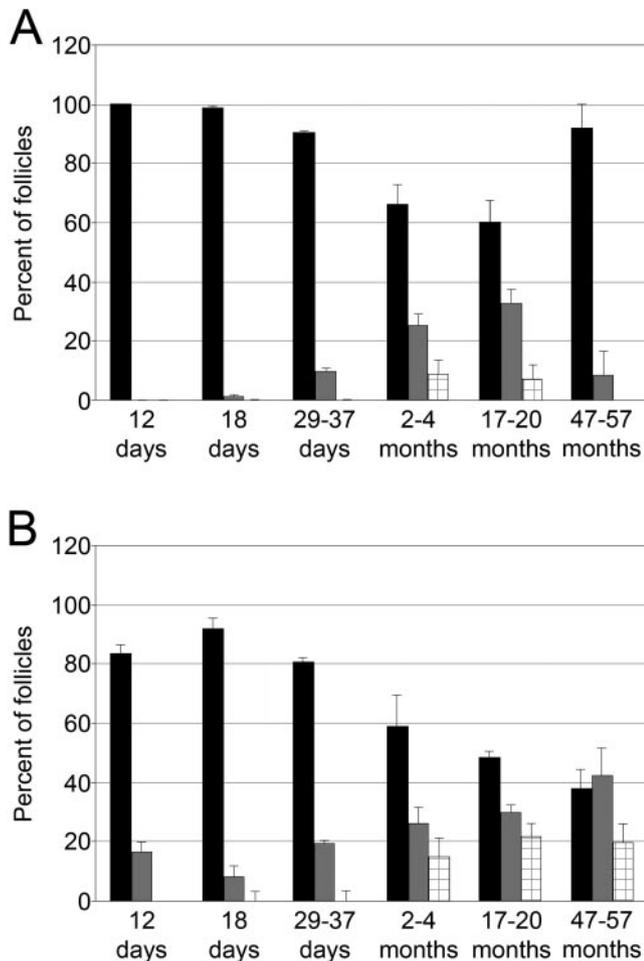


FIGURE 4. The approximate diameter of conjunctival follicles with relation to age is illustrated for (A) superior and (B) inferior conjunctivae (black bars: $<200\ \mu\text{m}$; gray bars: $200\text{--}300\ \mu\text{m}$; hatched bars: $>300\ \mu\text{m}$). In both regions, there was a steady progression in average follicular size, beginning at age 18 days. Follicle diameters greater than $300\ \mu\text{m}$ were not seen until 2 to 4 months of age. In the superior conjunctiva, most of the follicle diameters remained less than $200\ \mu\text{m}$ at all ages, while in the inferior conjunctiva, most of the follicles were greater than $200\ \mu\text{m}$ by 17 to 20 months. The larger, more robust follicles in the inferior conjunctiva, combined with their greater overall number in this region, suggests greater antigenic stimulation in this half.

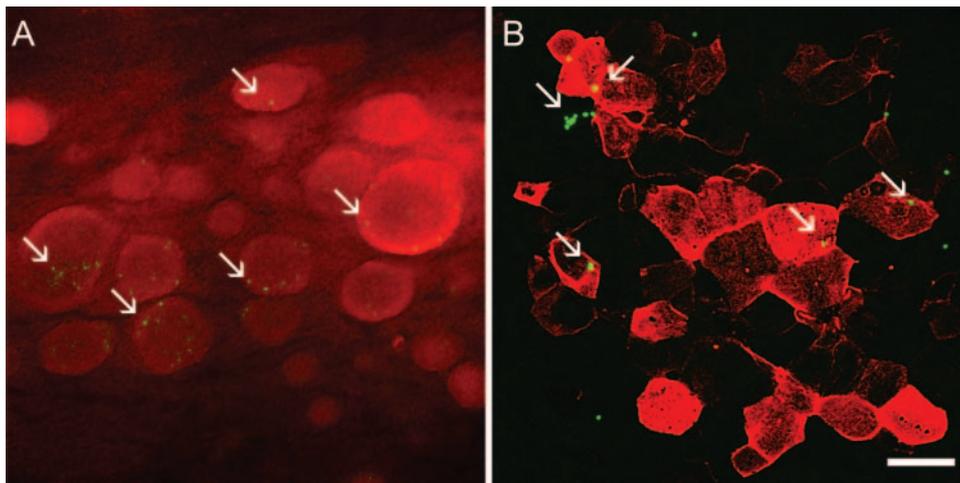


FIGURE 5. (A) Stereomicroscopic image of a cluster of inferior conjunctival follicles from a 2.3-month-old rabbit after a 50-minute in vivo exposure to $0.5\ \mu\text{m}$ green fluorescent latex beads. Individual beads and clusters of beads (arrows) were preferentially bound to follicle regions. (B) A two-dimensional maximum projection of an 80-step z -series extending $35.55\ \mu\text{m}$ down from the apical surface of a follicle in an 18-day-old animal. The surface of a select population of follicle-associated epithelial cells was labeled by UEA-biotin (red). The confocal z -series confirmed that the green fluorescent latex beads were in optical planes below the UEA-stained surface of the epithelium. Bar, $20\ \mu\text{m}$.

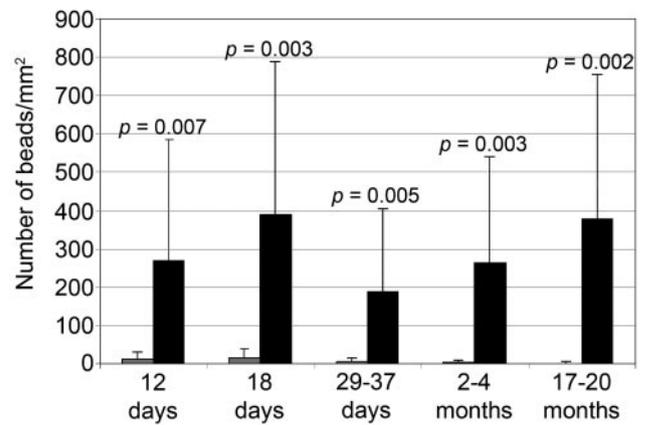


FIGURE 6. The uptake of fluorescent latex beads was evaluated using confocal laser scanning microscopy. This graph illustrates the average number of beads per square millimeter (mean \pm SD) in or directly below the follicle-associated epithelium (black bars) and control non-follicular palpebral epithelial cells (gray bars) for each age group after 67.7 ± 4.4 minutes of in vivo exposure. Bead uptake was significantly higher in the follicle-associated epithelium than in the nonfollicular control regions in each age group. Probabilities above each set of bars show the results of a t -test comparison. An ANOVA comparison of all follicle-associated epithelial data showed no significant difference ($P = 0.39$) in uptake at the different ages.

In humans, Peyer's patches are present at birth, then increase in number and size for at least the first 10 years of life, before a rapid decline by age 20, followed by a slower decline for the remainder of life, but are never fully lost.³ Bronchus-associated lymphoid tissue is present in 40% of individuals younger than 20 years, but only rarely in older cases.⁸ Similarly, larynx-associated lymphoid tissue was found in 80% of individuals younger than 20 years but only 56% of those older than 20 years.⁸ Salivary duct-associated lymphoid tissue in monkeys is absent in newborns, peaks by 1 year of age, and then sharply declines with increasing age.⁴ Larynx-associated lymphoid tissue is found in 100% of children less than 10 years of age before beginning a slow, gradual decline to 7.1% of those in their sixth decade of life. Age-dependent variation in human tear duct-associated lymphoid tissue (TALT) have been found in two studies. In this tissue, the frequency of O-TALT slowly increases between the age of 20 to a maximum at approximately 60 years of age before slowly declining with further aging.^{27,28}

Of interest, there is a loss of TALT associated with scarring due to symptomatic dacryostenosis, which raises the possibility that a limited exposure to antigens, as a consequence of reduced tear flow, resulted in downregulation of the lymphoid tissue.²⁸ Newborn human infants have no detectable conjunctival follicles at birth; however, an average of 27.1 follicles are found in 100% of children between 1 and 10 years of age, followed by a more sporadic appearance with increasing age.⁹ Approximately 60% of individuals have an average of 10.3 follicles/eye in their mid-70s.¹⁸ Age-related changes in rabbit O-CALT, therefore, mirror previously reported changes in human O-CALT and O-MALT in other tissue locations.

Paulsen et al.²⁹ have hypothesized that CALT and the closely related TALT play a role in the pathogenesis of dry eye. The present study is consistent with such a hypothesis, since the prevalence of dry eye is widely recognized to increase with age.³⁰ If the environmental antigens and pathogens normally encountered by the ocular mucosa overwhelm age-diminished O-CALT, subtle shifts in the balance of Th1 and -2 type responses or in the production of T-regulatory cells may trigger an inflammatory cascade leading to dry eye. Future studies should examine whether reduced levels of O-CALT in young children and the elderly contribute to the higher prevalence of ocular diseases, such as dry eye and conjunctivitis, which occur at these age extremes. In addition, the potentially adverse influence of immunomodulatory medications used to treat common ocular diseases, such as steroids or cyclosporine in the treatment of dry eye, on O-CALT needs to be characterized. Finally, the number of mucosal follicles and M cells in the intestine are known to change in response to commensal and pathogenic bacteria, and similar studies are needed on bacteria-related changes in the ocular mucosa.^{31,32} The present study established the rabbit conjunctiva as an ideal model for these studies.

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