DYNAMICS OF GOBLET CELLS DURING INFECTION BY *P. multocida* AND *B. bronchiseptica* IN RABBITS

Doncel B[1], Iregui CA[2], Botero L[3], Martínez N[2]

[1]Department of Sciences for Animal Health, Veterinary Pathobiology Research Group
[2]Department of Animal Production
Facultad de Medicina Veterinaria y de Zootecnia,
Universidad Nacional de Colombia, Bogotá, Colombia, South America

ABSTRACT

The number and histochemical characteristics of goblet cells (GC) in the nasal cavity and nasopharynx of healthy rabbits and rabbits suffering septicemia and rhinitis caused by *Pasteurella multocida* and *Bordetella bronchiseptica* were analyzed. Alcian blue and Periodic Acid Schiff histochemical technique were used. The results showed that the GC population remained constant in healthy rabbits during different stages of development, ranging from 50-57 ±25 GC/mm of the basal lamina. The GC number was determined in the maxilloturbinates (63 ±24GC/mm), nasal septum (53 ±18GC/mm) and nasopharynx (45 ±22GC/mm). Acid glycoproteins were predominant in all developmental stages, all anatomic regions and in sick animals. Rabbits suffering septicemia had a highly significant increase ($P<0.001$) in the number of GC compared to healthy rabbits and rhinitic animals. This study demonstrated increased GC number and secretions of acid glycoproteins during the respiratory disease of rabbits.

Key words: Rabbit, nasal cavity, respiratory disease, goblet cell, histochemical characterization, glycoproteins.

DINÁMICAS DE LAS CÉLULAS CALICIFORMES DURANTE LA INFECCIÓN POR *P. multocida* AND *B. bronchiseptica* EN CONEJOS

RESUMEN

Se evaluó el número y las características histoquímicas de las células caliciformes (CC) en la cavidad nasal y la nasofaringe de conejos sanos y enfermos de septicemia y rinitis inducida por *P. multocida* y *B. bronchiseptica*. Se usó la técnica histoquímica de azul de Alciano y ácido Periódico de Schiff. Los resultados mostraron que la población de CC en ambas regiones anatómicas permaneció constante durante las diferentes etapas del desarrollo de los conejos sanos, y estuvo entre 50-57 ±25 CC/mm de lámina basal. Se determinó el número de CC en el maxilocornete (63 ±24 CC/mm), en el septo nasal (53 ±18 CC/mm) y la nasofaringe (45 ±22 CC/mm). En todas las etapas del desarrollo de los conejos, regiones anatómicas y animales enfermos hubo predominancia de glicoproteínas ácidas. Los conejos septicémicos mostraron un incremento altamente significativo ($P<0,01$) en el número de CC comparado con los animales sanos y aquellos que sufrían de rinitis. Este estudio demostró

1 bdonceld@unal.edu.co
2 cireguic@unal.edu.co
3 lboteroe@unal.edu.co

un incremento en el número de CC y secreción de glicoproteínas ácidas durante la enfermedad respiratoria de los conejos.

Palabras clave: conejo, cavidad nasal, enfermedad respiratoria, células caliciformes, caracterización histoquímica, glicoproteínas.

Pasteurella multocida (*P. multocida)* and *Bordetella bronchiseptica (B. bronchiseptica)* induce disease in rabbits and some clinical forms include rhinitis and septicemia (4, 14). Previous studies also demostrated lung lesions (4, 12). The natural disease (6) and the experimental infection (1) showed increased activity of goblet cells (GC) and infiltration of polymorphonuclear neutrophils (PMN) in the nasopharynx and nostrils. It is well known that GC and PMN are usually involved in infectious, endotoxic and allergic processes (10, 22). GC hyperplasia, metaplasia, hypertrophy, and PMN activation are also induced in intra-tracheal instillation of lipopolysaccharide (LPS) in rats (8, 22, 23).

In addition, GC produce a viscoelastic mucus layer covering the epithelial cilia of the respiratory tract, also called the mucociliary apparatus (21). The mucociliary apparatus traps inhaled particles, transports them in an oral or aboral direction and eliminates them by transporting them to the oropharynx where the material is swallowed (20).

The purpose of this work was to describe the morphology, quantity, and the histochemical properties of the GC in healthy rabbits and diseased rabbits infected with *P. multocida* and *B. bronchiseptica* during two clinical forms of natural disease: rhinitis and septicemia.

MATERIALS AND METHODS

ANIMALS

New Zeeland White rabbits were selected from a conventional commercial farm and divided into 5 groups according to different developmental stages and health status. In healthy rabbits, there was a difference of 2 days between each one, beginning from one to 69 days. Rabbits from group 3 are more susceptible to the disease (11) (Table1). Nasal cavity, trachea and lungs were sampled for isolation of *P. multocida* and *B. bronchiseptica;* the samples were cultured on BHI (brain heart infusion) media containing 5% (v/v) defrinated sheep`s blood. The isolates were examined with biochemical tests (Api20, bio mèrieux^{R} sa, Marcy I´Etoile, France) and classified accordingly. No bacteria could be isolated from healthy rabbits, while in the groups of diseased animals *P. multocida* and/or *B. bronchiseptica* were isolated (Table 1).

Table 1. Groups, number, clinical and microbiological features of rabbits.

	No. animals	Age (days)	Condition	P. multocida and/or B. bronchiseptica isolation
Group 1	11	1-21	Healthy, suckling	-
Group 2	14	23-49	Healthy, weaning	-
Group 3	10	51-69	Healthy, fattening	-
Group 4	10	51-69	Sick, rhinitis	+
Group 5	10	51-69	Sick, septicemia	+
Total	55			

Tissue fixation

Rabbits were intramuscularly anaesthetized with 35 mg/Kg ketamine hydrochloride (Rotexmedica, AG, Germany) and 5 mg/Kg xylazine hydrochloride (Bayer, Triitau, Germany), followed by intranasal instillation of paraformaldehyde (MERCK, Darmstadt, Germany) 4% - glutaraldehyde (POLYSCIENCES.INC, Warrington, PA) 0.5% solution. Euthanasia was performed by means of an overdose of anesthesia. The nasopharynx was dissected and a 0,5 cm thick cross-section of the nostril (in front of the first molar) was obtained and postfixed in 3,7% buffered formaldehyde. Tissues were decalcified in a 7% EDTA disodium salt solution.

Goblet cell histochemistry using the AA/PAS technique

GC were stained using a modified Alcian blue (POLYSCIENCES.INC, Warrington, PA)-periodic acid Schiff (AA/PAS) histochemical technique (2). Briefly, tissue sections (3 μm) were immersed in 3% acetic acid, drained, submerged in 1% AA, pH 2,5, for 30 minutes, washed in distilled water and then immersed in 0.5% periodic acid for 5 minutes. Subsequently, the samples were rinsed in distilled water and Schiff reagent was then added (PAS reaction) for 15 minutes. The reaction was then rinsed in 4,5% sodium pyrosulphate for 50 seconds. Lastly, the slides were washed in running water for 20 minutes. GC containing acid glycoproteins stained blue or purple and were called AA^+. GC containing neutral glycoproteins colored magenta or violet and were called PAS^+. GC staining blue or purple and magenta or violet were called AA/PAS^+ (21). The sections were randomly labeled by a technician who was not involved in the work to facilitate a blind evaluation.

Morphological examination

Qualitative variables analyzed were GC size change, number in the nasal cavity and nasopharynx and the presence of secretions in the nasal cavity (Table 2).

Table 2. Morphological evaluation parameters of GC and their secretions in rabbits.

Variable/ response	Small	Large	
GC size	Pushing or slightly pushing the cytoplasmatic membrane of epithelial cells neighboring GC	GC pushing neighboring epithelial cells which caused the displacement to be greater than 50% of their diameter	
	Basal level (0)	**Moderate (+)**	**Severe (++)**
GC number	One GC per 5 ciliate epithelial cells	One GC per 2 ciliate epithelial cells	2 or more GC per 1 ciliate epithelial cell
Secretions in the nasal cavity	None or minimum accumulation of secretions randomly distributed in small spots on the epithelium of the nasal septum and maxilloturbinate	Accumulation of secretions randomly distributed in lumps around the nasal septum and maxilloturbinate	Accumulation of secretions randomly distributed in compact form around the nasal septum and maxilloturbinate

Histometry

The number of GC/mm of basal lamina in the nostril and nasopharynx was determined microscopically by counting the cells at high power magnification (400X) coupled with an image analyzer (Leco image analysis system, version 2,02). The nostril respiratory epithelium was divided into the nasal septum and the maxilloturbinates, 32 and 12 optical fields from each subdivision were measured, respectively (Fig.1). Six to 15 optical fields were counted in the nasopharynx.

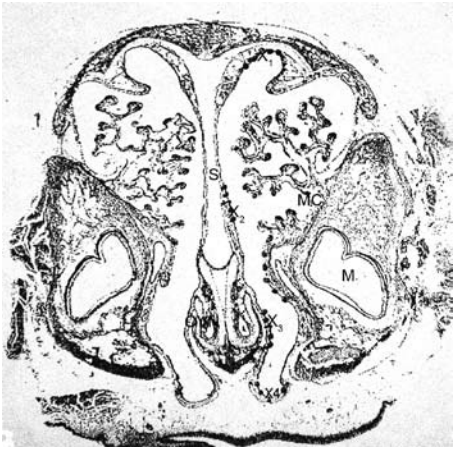

Figure 1. Rabbit nostrils. Cross section. Reference points and optical fields evaluated in the histometric analysis. $X_{(1, 2...)}$: reference points to begin quantifying GC; • optical fields evaluated. The evaluation was done at 400X magnification. M: molar; OVN: vomeronasal organ; S: septum; MC: maxilloturbinate.

Statistical analysis

The morphological variables analyzed in each group were GC size change, GC number and the presence of secretions in the nasal cavity, based on previous studies (17). Frequency histograms were used to graphically compare data for each variable between groups and anatomical regions using the chi square and maximum verisimilitude tests (SAS 6,12, SAS Institute Inc., Cary, NC, USA. 1996). In the quantitative analysis of total number of GC/mm of basal lamina, and relative quantities of AA+, PAS+ and AA/PAS+ GC populations in the respiratory epithelium of the nasal septum, data collected from the maxilloturbinate and nasopharynx were analyzed using a 3X3 factorial design. ANOVA was used for comparing groups and the Tukey test was used for comparing anatomical regions (SAS 6,12, SAS Institute Inc., Cary NC, USA. 1996). Independent comparisons between healthy groups (1, 2 and 3) and between susceptible healthy (group 3) and diseased groups (groups 4 and 5) were also performed. In this test the total GC/mm was the non parametric variable, and a Kruskal-Wallis non-parametric two tails test was employed. Differences were considered to be highly statistically significant and statistically significant when $p<0,01$ and $p<0,05$, respectively.

RESULTS

Morphological examination

The accumulation of mucus in nasal cavity lumen was the only statistically significant variable. Group 1 had a greater percentage of animals with secretions at the basal level in the nasal cavity in comparison to group 2 ($p<0,05$), whereas the difference was highly significant ($p<0,01$) compared to the other groups (Table 3). Groups 1 and 2 had highly significant less percentage of animals having moderate secretions compared to groups 3, 4 and 5 ($p<0,01$).

Animals in groups 3, 4 and 5 had large amount of mucus that was significantly higher than the amount of mucus from groups 1 and 2 ($p<0,01$). Even though no differences were detected among animals in groups 3, 4 and 5, it was evident that the amount of mucus present in sick rabbits (groups 4 and 5) was greater than the amount of mucus found in animals in group 3.

Table 3. Comparison of percentage of animals with different degrees of accumulation of secretions in the nasal cavity between rabbits groups 1, 2, 3, 4 and 5. Statistical differences: ac, cd, cf and hi p<0.05. ab, cg, hj, ij and kl p<0.01.

Grupo Grade	1	2	3	4	5
Basal level	95a	71c	35bd	40bf	30bg
Moderate	5h	29i	50j	35j	45j
Severe	0k	0k	15l	25l	25l

HISTOMETRY

Number of GC and histochemical properties in healthy rabbits by groups

There were no significant differences between the number of GC of the respiratory epithelium of the nasal cavity and the nasopharynx of healthy rabbits among any group (Table 4). Based on histochemical characteristics, group 2 had significantly fewer AA+ GC cells (19 ±10 GC/mm) in comparison to group 3 (23 ±15 GC/mm). Also, group 1 had highly significant fewer AA/PAS$^+$ GC cells (19 ±11GC/mm) than group 2 (25 ±14 GC/mm). In all groups, AA$^+$ and AA/PAS$^+$ cell number was always larger than the number of PAS$^+$ cells.

Number of GC and histochemical properties in healthy rabbits by anatomical regions

The total number and PAS$^+$ GC in the maxilloturbinate were highly significant more abundant than those in nasopharynx (p<0.01) (Table 5). The number of AA$^+$ and AA/PAS$^+$ GC was always larger than the number of PAS$^+$ GC in all regions.

Table 4. Total number and relative quantities of GC according to their histochemical characteristics in nostrils and nasopharynx from 1-5 groups of rabbits. * significant differences (p<0.05) and ** highly significant differences (p<0.01).

Group GC	1	2	3	4	5
Total	50 ±22	57 ±25	53 ±18	57 ±26	69 ±21 □
AA$^+$	22 ±12	19 ±10*	23 ±15*	26 ±18	27 ±11
PAS$^+$	8 ±10	13 ±14	9 ±8	10 ±9	13 ±10
AA-PAS$^+$	19 ±11**	25 ±14**	21 ±10	21 ±11	28 ±13 □

□ p<0.05, 5 vs 3 y 4 groups. □ p<0.01, 5 vs 3 y 4 groups

Table 5. Total number and relative quantities of GC according to their histochemical characteristics from anatomic regions from healthy rabbits. * significant differences (p<0.05) and ** highly significant differences (p<0.01).

Region GC	Septum	Maxilloturbinate	Nasopharynx
Total	53 ±18	63 ±24**	45 ±22**
AA^+	19 ±9	22 ±15	22 ±14
PAS^+	11 ±8	15 ±15**	6 ±8**
AA-PAS^+	24 ±10	26 ±14	17 ±11

Number of GC and histochemical properties in susceptible healthy vs. sick rabbits by groups

The total number of GC and the number of AA/PAS^+ GC in group 5 were significantly larger than those in groups 4 and 3, p<0,01 and p<0,05, respectively (Table 4). Group 5 had the highest total number of GC (69 ±21 GC/mm), followed by group 4 (57 ±26 GC/mm) and group 3 (53 ±18 GC/mm). Also group 5 had the highest number of AA/PAS^+ cells (28 ±13), followed by group 4 (21 ±11) and group 3 (21 ±10). In all groups, the number of AA^+ and AA/PAS^+ GC was always greater than the number of PAS^+ GC.

Number of GC and histochemical properties in susceptible healthy vs. sick rabbits by anatomical regions

The total number of GC and AA^+ GC in all groups was significant larger in the maxilloturbinate than in other regions (p<0,01) and (p<0,05), respectively (Table 6). In all regions, the number of AA^+ and AA/PAS^+ GC was always larger than the number of PAS^+ GC.

Table 6. Total number and relative quantities of GC according to their histochemical characteristics from anatomic regions from 3, 4 and 5 groups of rabbits. * significant differences (p<0.05) and ** highly significant differences (p<0.01).

Region GC	Septum	Maxilloturbinate	Nasopharynx
Total	58 ±17	69 ±27**	52 ±21**
AA^+	22 ±11	31 ±18*	22 ±13
PAS^+	11 ±8	12 ±11	9 ±10
AA-PAS^+	25 ±10	25 ±14	20 ±10

DISCUSSION

To better understand the possible role of rabbit GC in septicemia or rhinitis induced by *P. multocida* and *B. bronchiseptica*, the basal level and the biochemical properties of GC in the upper respiratory airways of healthy and diseased animals was established. The results revealed that the GC of healthy rabbits from different age groups ranged from 50 to 57 ±25 cells/mm in the upper respiratory epithelium with no significant differences between groups (Table 4). Conversely, the number of GC in healthy rabbits was highly significantly different ($p<0,01$) between the maxilloturbinate and the nasopharynx. These values correspond to the basal level of GC under physiological conditions.

Regardless of age and anatomical region, healthy rabbits have larger numbers of both GC with acid glycoproteins (AA^+) and GC with acid and neutral glycoproteins (AA/PAS^+) than the number of GC that contain neutral glycoproteins (PAS^+) alone (Tables 4 and 5). Similar findings were reported in the tracheo-bronchial epithelium of rabbits (18) and in the nasal, paranasal and nasopharynx epithelium of healthy monkeys (*Macaca radiata*) (9). The results of our studies revealed minimal differences on the biochemical properties of GC between different regions of the respiratory epithelium of healthy rabbits; such properties are similar to those of the GC of animal species that present extremely different lifestyles.

There was a highly significantly larger goblet cell population in rabbits suffering from septicemia compared to those animals suffering from rhinitis or susceptible healthy rabbits ($p<0,01$) (Table 4). Based on these results, it can be argued that the increase in the quantity of GC would be due to hyperplasia or metaplasia in acute respiratory pathologies of the rabbits (particularly in the septicemic form, and the rhinitic form to a lesser extent). However, such increase was not excessive in absolute terms, and the variance of this increase in all examined groups was high (69 ±21 GC/mm in septicemic animals, 57 ± 26 GC/mm in rhinitic animals and 53 ±18 GC/mm healthy animals). In addition to the two proposed causes of increased number of GC (hyperplasia and/or metaplasia), it would be precautious to take into account that some apparent variations in the number of GC could be more the result of changes in their functional state rather than changes in the actual number of GC. Functional variations in GC secretory stages - such as hypersecretion - may alter the count of such cells. Since GC become more evident during their hypersecretory stage, it is easier to identify them by light microscopy. In contrast, when their secretion has been completely excreted, GC are smaller; thus, their identification can be more challenging. As a result, the number of cells counted does not represent the actual population size. In addition, the functional variations mentioned above could be corroborated by the fact that the greater accumulation of mucus secretion was present in the lumen of the nostrils in septicemic and rhinitic rabbits. Thus, it is suggested that in addition to absolute increases in the number of GC (hyplerplasia) or relative increases (metaplasia) there would also be important changes in cell activity (hypersecretion) in acute rhinitis or septicemia in rabbits. Therefore, such functional change has repercussions on the apparent quantitative variation of these cells when some studies do not employ morphometric techniques. In other words, some interpretations of hyperplasia that appear in the literature (1, 25) could actually be dealing with secretory physiological changes. Fluctuations in percentages of GC according to their histochemical properties in rabbits affected by rhinitis or septicemia would coincide with the physiological stages since there were no variations

in regards to percentages of subpopulations in healthy animals.

In terms of host survival, it does not seem to be advantageous for the respiratory system, particularly in the larger airways, to respond with a great number of GC in acute pathological conditions. In such conditions, the host runs the potential risk of blocking its airways when a large number of GC secrete larger quantities of mucus. This would also be a disadvantage in terms of the mucociliary apparatus since a greater number of GC, a larger size and/or a greater quantity of mucus secreted cause an overload of the ciliated cells as a result of the excess of secretion; therefore, an obstructive clinical picture will be developed. Rogers (20) reported that goblet cell metaplasia in small airways with subsequent hypersecretion can readily compromise mucociliary transport which in turn leads to mucus trapping and airflow obstruction. When comparing this process with the acute inflammatory response, in which the equilibrium between pro and anti-inflammatory phenomena is kept under control, it is observed that the GC response trends would establish an equilibrium during the acute phases of the disease due to the reasons mentioned above. In chronic respiratory processes an overbalance of GC has been described, just as it occurs in a chronic inflammatory responses; thereby, the typical hyperplasia previously described can be found (15, 25).

In spite of the autonomous response shown by GC in infectious and/or inflammatory processes of the airways, such response seems to be closely linked to the presence and activation of PMNs which are stimulated by LPS of Gram (-) bacteria. Komatsu *et al*, Long *et al* y Shimizu *et al* (13, 16, 22) have reported the same association. Experimental administration of LPS in different animal species by intra-tracheal or intra-nasal route causes not only hyperplasia or metaplasia of GC in different regions of the respiratory tract but also additional infiltration of PMN (7, 22, 23, 24, 25). Voynow reported metaplasia of GC in lung airways of mice that had received human neutrophyl elastase by oropharyngeal aspiration and concluded that neutrophyl elastase proteolytic activity initiates an inflammatory process that leads to goblet cell metaplasia. A similar relationship has been proposed between these two types of cells in a previous work conducted using the same rabbits employed in this study (6).

This study both described the population of GC and their biochemical characteristics in upper airways of healthy rabbits and lead us to suggest that goblet cells participate actively in the course of respiratory disease in rabbits. Such participation means that just as there is a moderate increase in their number, perhaps more importantly, there is a hyperactivity response that it is reflected as a hypersecretion and possibly a greater synthesis of acid glycoproteins. Bearing in mind the survival of affected animals, it would not seem to be a useful strategy to severely increase the number of GC during acute phases of respiratory disease. The LPS derived from microorganisms most frequently isolated from this pathology (*P. multocida* and *B. bronchiseptica*) as well as the bacteria themselves, would play a decisive role in activating GC, possibly mediated by PMNs.

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