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EFFECT OF RN-301 IMMUNOMODULATOR ON BRONCHUS-ASSOCIATED LYMPHOID TISSUE (BALT) IN PROTEIN DEPLETED RATS AT WEANING

MARIA GABRIELA MARQUEZ, GUSTAVO A. SOSA, NORA H. SLOBODIANIK[1], ALEJO FLORIN-CHRISTENSEN[2], MARIA ESTELA ROUX

Laboratorio de Inmunología Celular, Departamento de Ciencias Biológicas, y [1] Departamento de Nutrición, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires; [2] Centro de Educación Médica e Investigaciones Clínicas (CEMIC), Buenos Aires

Summary It has been previously demonstrated in Wistar rats that severe protein deprivation at weaning, even after refeeding with a 20% casein diet for 21 days, provokes alterations in IgA+ B cell and T cell populations from gut and GALT (gut associated lymphoid tissue) that are reverted by immunomodulator IM-104. In the present report, we investigate the influence of RN-301 (quite similar to IM-104) given by the oral or subcutaneous route during the protein deprivation period, in the seeding of BALT with IgA+ B and CD5+T cells. The immunomodulator RN-301 contains LPS from *E. coli* and membrane and ribosomal fractions of *P. acne*. Tissue sections of the lower respiratory tract were studied by immunohistochemistry. The immunomodulator RN-301 administered by the oral route favours the significant increase in the seeding of the BALT lamina propria with IgA+B and CD5+T cells ($p < 0.001$). However, the RN-301 given by the subcutaneous route does not favour the repopulation of the BALT lamina propria. The ribosomal fractions from *P. acne* associated with LPS from *E. coli* contained in the immunomodulator RN-301 administered by the oral route may rescue the small resting lymphocytes in the gut-associated lymphoid tissue (GALT). This event favours their proliferation and migration to the BALT.

Key words: malnutrition, immunomodulator RN-301, BALT

Mucosae interface with the environment and are constantly exposed to antigens of microbial origin. Moreover, the intestinal mucosa is exposed to antigens derived from food and the respiratory mucosa is exposed to inhaled antigens. Therefore, the mucosal lymphoid tissues fulfill several functions necessary for immunological protection.

The presence of organized collections of lymphoid tissues, which have been termed MALT (mucosa-associated lymphoid tissues) is a characteristic of mucosal surfaces. The nodular tissues of GALT (gut-associated lymphoid tissue) and their analogs in bronchus-associated lymphoid tissue (BALT) consist of lymphoid follicles found singly in the oral mucosa, intestine and bronchi or in clusters in the nose-associated lymphoid tissue of mice and rats and in the tonsils, adenoids, Peyer's patches, appendix[1]. BALT in the rat is localized along the main bronchi, near the bifurcations in all lung lobes[2-5].

Malnutrition, and specially protein deficiency, provokes an impairment of cell-mediated immunity, immune responses and secretory antibody responses[6].

Recent studies from our laboratory in Wistar rats have shown for the first time, the permanent damage that severe protein deprivation at weaning induces in BALT[7].

In previous works we have shown that an immunomodulator (IM-104) orally administrated during the protein deprivation period promotes cellular differentiation and maturation in GALT and a

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Dirección postal: Dra. María Estela Roux, Ugarteche 3050, 1425 Buenos Aires, Argentina.

good repopulation of gut lamina propria with IgA B cells when rats were refed with a 20% casein diet[8].

The aim of the present work was to investigate the influence of the immunomodulator RN-301 orally and subcutaneously administered to Wistar rats during the protein deprivation period, in the seeding of BALT with IgA B and T cells after protein refeeding.

Materials and Methods

Animals and Diets: Weaning rats of the Wistar strain (closed colony from the breeding unit kept at the animal facilities of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Argentina) of either sex, 21-23 days old, were fed ad libitum a protein free diet until they lost 25% of their initial body weight (36-39 days of age) and afterwards were fed on a 20% casein diet for 21 days (57-60 days old) (R21). Simultaneously, a group of weaning rats were fed a protein-free diet together with the immunomodulator RN-301 dissolved in the drinking water– so that animals received 1 mL/Kg of body weight daily– until they lost 25% of their initial body weight (36-39 days of age), and another group of weaning rats were fed a protein free diet and received the same amount of the immunomodulator RN-301 by subcutaneous injection at days 1-3-7 during the same protein free diet period (36-39 days of age); both groups of rats were refed a 20% casein diet for 21 days (57-60 days old) (RN-R21 and RN-R21SC respectively).

Age-matched well nourished control group receiving stock diet (Cargill, Argentina, 24.6% protein) since weaning was run simultanesously (57-60 days of age) (C).

During all the experiments the animals were exposed to a 12 hour light-darkness cycle, room temperature was kept at 21°C ± 1.0.

Water and diets were offered ad libitum.

Experimental isocaloric diets were prepared as previously reported according to Farina et al.[9]: to a basal concentrated diet containing all the essential nutrients – as recommended by the American Institute of Nutrition[10]– except protein, casein was incorporated to provide 20% protein and then filled up to 100 g by adding dextrin. In the protein free diet casein was omitted and replaced by dextrin.

Immunomodulator RN-301: Contains per mL 0.93 µg of lipopolysaccharide (LPS) from *E. coli* and 0.168 g of membranous and ribosomal fractions from *Propionibacterium acne* expressed as ribose in suspension (Neomar SRL).

Tissue Sections: Experiments were performed using 5 animals. Rats were sacrificed after 4 hours fasting, and the lower respiratory tract was removed, placed in ethanol 4°C in order to be processed by Sainte Marie's technique[11]. Briefly, tissues were fixed in 95% ethanol pre-cooled at 4°C, dehydrated in 4 changes of pre-cooled absolute ethanol, cleared by passing through 3 consecutive baths of pre-cooled xilene and embedded in paraffin at 56°C. Sectioning was carried out as usual, and tissue sections (4-5 µm thick) were placed on glass slides. Paraffin was removed by gently motioning the slides in 2 consecutive baths of xylene, which was removed in 3 baths of saline solution (0.9% w/v in distilled water).

Tissue sections of the lower respiratory tract were studied with an Olympus fluorescence microscope. Number of cells per 15 fields (mean ± SE) in the lamina propria of BALT were recorded in each tissue section. All slides were reviewed by two blinded investigators. B and T cells were labelled by an indirect immunofluorescence technique with the following antibodies: 1) B cells in tissue sections were labelled with IgG affinity purified goat against rat IgA (α chain specific) (Organon Teknika Corporation) followed by fluorescein conjugated $F(ab)'_2$ fraction of rabbit against goat IgG (H and L) (Organon Teknika Corporation); 2) T cells in tissue sections were labelled with the xenogeneic monoclonal antibody (mAb OX19) against rat CD5 antigen expressed on all thymocytes and peripheral T cells but not on B cells, macrophages or NK cells (Accurate Chemical & Scientific Corp., Westbury, NY); followed by the fluorescein conjugated goat $F(ab)'_2$ fragment to mouse IgG (whole molecule) (Organon Teknika Corporation).

Results are expressed as number of cells in 15 fields ± SE, and 2 or 3 sections per animal were recorded.

Statistical analysis was performed by using analysis of variance (ANOVA) and Tukey-Kramer, taking $p < 0.05$ as significant.

Results

Tissue section study of each experimental group compared to the control showed no variation in BALT localization between them. However, serial BALT tissue section study points out a significant decrease in the size and number of organized collection of lymphoid tissue in the R21 group when compared to the control C. This observation was accompanied by a decrease in the BALT lamina propria cellularity (Figures 2 vs 1). When tissue sections from rats treated with the immunomodulator given by the oral route (RN-R21) were compared to rats receiving only 20% casein diet during 21 days (R21), an increase in lamina propria cellularity was observed (Figure 3 vs 2) although it did not reach the cellularity found in control rats (Figure 3 vs 1). However, the quantity and size of organized collection of lymphoid

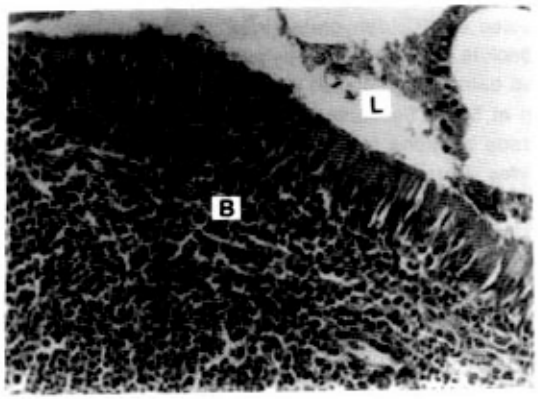

Fig. 1.– B = BALT L = lumen
BALT from control rats (Hematoxilin-Eosin, 150X)

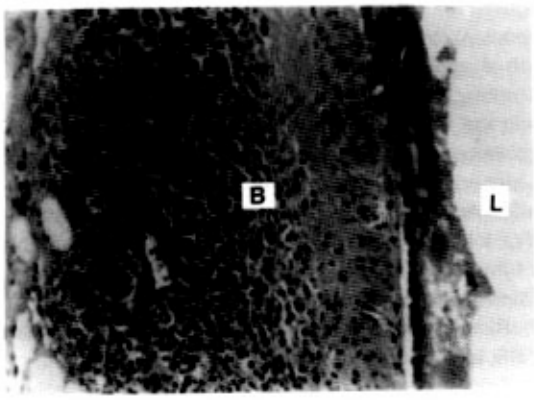

Fig. 2.– BALT from refed rats with 20% casein diet during 21 days (Hematoxilin-Eosin, 150X). A significant decrease in the lamina propria cellularity was observed in the R21 group compared to the control C (Figure 2 vs 1).

tissue in RN-R21 (rats receiving immunomodulator RN-301) could not reach the one found in rats receiving stock diet (C, control animals).

Table 1 describes the number of IgA+B and CD5+T cells found in the BALT lamina propria from protein refed, protein refed treated with RN-301 and control rats. The number of IgA B cells was significantly diminished in the protein refed rats (R21) as well as in the protein refed rats subcutaneously treated with RN-301 (RN-R21SC) when compared to the control (RN-R21SC vs R21 vs C, $p < 0.001$). However, when rats were orally treated with the immunomodulator RN-301 in the drinking water during the protein deprivation period, the number of IgA B cells in RN-R21 group attained control number and was significantly different from R21 group (without immunomodulator)

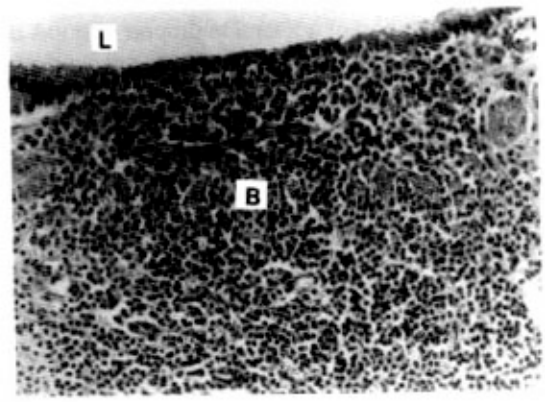

Fig. 3.– BALT from rats treated with RN-301 administered by the oral route during the protein deprivation period and refed with 20% casein diet during 21 days (Hematoxilin-Eosin, 150X). An increase in lamina propria cellularity was observed when comparing FIGURE 3 vs 2, although it did not reach the cellularity found in control rats (Figure 3 vs 1).

TABLE 1.– *IgA B and CD5 T cells in BALT lamina propria from protein refed, protein refed treated with RN-301 and control rats*

Number of cells in 15 fields per section (mean ± SE)		
Group	B cells IgA+	T cells CD5+
C	397.20 ± 37.70*	465.86 ± 37.57#
R21	270.10 ± 22.50	291.64 ± 22.18
RN-R21	442.70 ± 29.60*	449.20 ± 24.76#
RN-R21SC	202.25 ± 11.40	184.10 ± 18.52

5 animals and 2 sections per animal were recorded; C: Control group; R21: Rats refed with 20% casein diet during 21 days; RN-R21: Rats orally treated with RN-301 during protein deprivation, refed with 20% casein diet during 21 days; RN-R21SC: Rats subcutaneously treated with RN-301 during protein deprivation, refed with 20% casein diet during 21 days; ANOVA C vs R21 vs RN-R21 vs RN-R21SC, $p < 0.001$

*Significantly different from the remaining groups ($p < 0.01$) but not between them.

#Significantly different from the remaining groups ($p < 0.001$) but not between them.

(RN-R21 vs R21, $p < 0.001$). The number of CD5+ T cells detected with the monoclonal antibody OX19 followed the behaviour described above for IgA B cells (RN-R21SC vs R21 vs C, $p < 0.001$ and RN-R21 vs R21, $p < 0.001$).

Discussion

In the present report we found that the oral treatment with the Immunomodulator RN-301 during the protein deprivation period leads to a recovery from the immunodeficient state increasing the seeding of BALT with IgA B and T cells after protein refeeding.

The lipopolysaccharide (LPS), a component of the RN-301, is an endotoxin of the external membrane of Gram negative bacteria. The LPS has been used to induce antibody response in the absence of helper T cells and besides other properties, it is also a mitogen for B cells[12-14]. Studies in a number of laboratories have shown that IgA antibodies have been induced to LPS and capsular polysacharides[15]. Moreover, work showing the appearance of IgA anti Salmonella LPS mAbs and protection against an oral Salmonella challenge has been described[16]. LPS is also very important in the regulation of B cell terminal differentiation, increasing the production of IL-4, IL-5 and IL-6 as it has been observed in nude mice[17]. A recent paper ascertained that bacterial crude extract or bacterial ribosomes are recognized similarly by mucosal B cells. Ribosomes appear to trigger stimulation of Peyer's patches and the subsequent spreading of sensitized B cells towards all mucosal areas[18].

The efficiency of RN-301 orally administered may be explained as follows: the severe protein deprivation provokes an arrest in B and T cell differentiation[19, 20]. The LPS as well as the ribosomal fraction from *Propionibacterium acne* – contained in the immunomodulator RN-301– stimulates the proliferation of the small resting lymphocytes found in the Peyer's patches, and their subsequent migration to mucosal associated lymphoid tissue such as BALT in the present study. It is known that the small B resting lymphocytes in the Peyer's patches that express IL-4R are ready to enter the cell cycle and to continue from G1 to S arriving to M and that resting TH cells express IL-2Rβ but when entering in G1 they express sIL-6R and secrete IL-6[21]. Moreover, IgA B cell terminal differentiation or maturation into IgA plasma cells needs IL-6[22] and the LPS also has the property of stimulating IL-6 production[22, 23]. Therefore, we think that the significant increase of IgA B and T cells in the BALT lamina propria of rats treated orally with the immunomodulator RN-301 during the protein deprivation period, is due to its LPS and ribosomal fraction from *Propionibacterium acne* content.

Our results demonstrate the importance of the oral route over the subcutaneous one. Only, the oral administration of RN-301 allows BALT lamina propria repopulation with large quantities of IgA+ B and CD5+ T cells. The immunomodulator RN-301 administered subcutaneously is not effective at the dose and form utilized. This observation is in agreement with previously published papers where the oral delivery of antigens, vaccines or immunomodulators is essential for the induction of mucosal immune responses at different sites[24-28].

Resumen

Efecto del inmunomodulador RN-301 sobre el tejido linfoide asociado a bronquios (BALT) en ratas que al destete sufrieron depleción proteica severa

Trabajos previos demostraron que la depleción proteica severa al destete en ratas Wistar provoca alteraciones permanentes en las poblaciones celulares B IgA+ y T del intestino delgado y del tejido linfoideo asociado a intestino (GALT) que son revertidas por el inmunomodulador IM-104; estos efectos persisten aún después de la recuperación nutricional con la administración de caseína al 20% durante 21 días. En el presente trabajo investigamos la influencia del inmunomodulador RN-301 (similar al IM-104) administrado por vía oral o subcutánea durante el período de depleción proteica, sobre la repoblación de la lámina propia (LP) del BALT (tejido linfoideo asociado a bronquios). El RN-301 contiene LPS de *Escherichia coli* y fracciones membranosas y ribosomales de *Propionibacterium acne*. Los cortes de tejido del tracto respiratorio bajo fueron estudiados por inmunohistoquímica. El RN-301 administrado por vía oral favorece en forma estadísticamente significativa la repoblación de la LP del BALT con células de B IgA+ y T CD5+ ($p < 0,001$). El RN-301 administrado por vía subcutánea no favorece la repoblación de la LP del BALT. La fracción ribosomal de *Propionibacterium acne* asociada con el LPS de *E. coli* constituyentes del inmunomodulador RN-301 administrado por vía oral rescatarían pequeños linfocitos en reposo del GALT estimulando su proliferación y favoreciendo su migración hacia el BALT.

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References

1. Phillips-Quagliata JM, Lamm ME. Lymphocyte Homing to Mucosal Effector Sites. *In:* Ogra PL, Lamm ME, McGhee JR, (eds). Handbook of Mucosal Immunology. New York: Academic Press 1994; 225-39.
2. Scicchitano R, Stanisz A, Ernest P, Bienenstock J. Common Mucosal Immune System Revisited. *In:* Husband AJ, (ed). Migration and Homing of Lymphoid Cells. Orlando: CRC Press 1988; 1-34.
3. Bienenstock J. Gut and Bronchus Associated Lymphoid Tissue: an overview. *In:* Nieuwenhuis P, Van Den Broek A, Hanna Jr (eds). Advances in Experimental Medicine and Biology. New York: Plenum Press, 1982; 149: 471-7.
4. Pabst R. Compartmentalization and kinetics of lymphoid cells in the lung. *Reg Immunol* 1990: 3: 62-71.
5. Plesch B. Histology and Immunohistochemistry of Bronchus Associated Lymphoid Tissue (BALT) in the rat. In: Nieuwenhuis P, Van Den Broek A and Hanna Jr, (eds). Advances in Experimental Medicine and Biology. New York: Plenum Press, 1982; 149: 491-7.
6. Watson RR and McMurray DN. The effects of malnutrition on secretory and cellular immune processes. *CRC Crit Rev. Nutr. Food Sci.* 1979; 12: 113-59.
7. Márquez MG, Sosa GA, Roux ME. IgA B and T lymphocytes in the bronchus associated lymphoid tissue of malnourished rats. *Com Biol* 1994; 12(2): 105-11.
8. Gonzalez Ariki S, Florin Christensen A, Roux ME. Efectos del Inmunomodulador-104 sobre la población linfocitaria de la mucosa intestinal de ratas inmunodeficientes. *Inmunología* 1993; 12(2): 59-63.
9. Farina R, Río ME, Sanahuja JC. A rapid method for evaluation of protein quality. *Nutr. Rep. Int.* 1977; 16: 202-97.
10. American Institute of Nutrition, Report of the Committee. *J Nutr* 1977; 107: 1340.
11. Sainte-Marie G. A paraffin embedding technique for studies employing immunofluorescence. *J. Histochem. Cytochem.* 1962; 10: 250-6.
12. Michalek SM, Kiyono H, Wannemuehler M, Mosteller LM, Mc Ghee JR Lipopolysacharide (LPS) regulation of the immune response. LPS influence on oral tolerance induction. *J. Immunol.* 1982; 128: 1992.
13. Mc Ghee JR, Michalek SM, Kiyono H, Babb JL, Clark MP, Mosteller LM. Lipopolysaccharide regulation of the IgA immune response. In: Strober W, Hanson LA and Sell KW, (eds). Recent Advances in Mucosal Immunity. N. York: Raven Press, 1982; 57-72.
14. Jacobs DM. Lipopolysaccharide and the Immune Response. In: Strober W, Hanson LA, Sell KW (eds). Recent Advances in Mucosal Immunity. N York: Raven Press, 1982; 47-56.
15. Abraham E, Robinson A. Oral immunization with bacterial polysaccharide and adjuvant enhances antigen-specific pulmonary secretory antibody response and resistance to pneumonia. *Vaccine* 1991; 9: 757.
16. Babb JL, Kiyono H, Michalek SM, McGhee JR. LPS regulation of the immune response: suppression of immune response to orally-administered T-independent antigen. *J Immunol.* 1981; 127: 1052.
17. Kawanishi H, Joseph K. IL-4, IL-5 and IL-6-mediated regulation of immunoglobulin (Ig) heavy chain class switching and Ig production by gut-associated lymphoid tissue (GALT) B cells from athymic nude (nu/nu) mice. *Immunological Investigation* 1991; 20(7): 605-21.
18. Zanin C, Béné MC, Perruchet AM, Perrin P, Martin F, Borelly J, Faure G. Bacterial crude extracts or ribosomes are recognized similarly by peripheral and mucosal B cells. *FEMS Immunology and Medical Microbiology* 1994; 10: 11-8.
19. López MC, Roux ME. Impaired differentiation of IgA-B cell precursors in the Peyer's patches of protein-depleted rats. *Dev. and Comp. Immunol* 1989: 13: 253-62.
20. Melton E and Roux ME. Detection of TdT-positive cells in the mesenteric lymph nodes of immunodeficient rats: study of their antigenic phenotype. *Reg. Immunol.* 1989; 2: 408-13.
21. Beagley KW, Eldridge JH, Lee F, et al. Interleukins and IgA synthesis. Human and murine Interleukin 6 induce high rate IgA secretion in IgA-committed B cells. *J Exp Med* 1989; 169: 2133-48.
22. Ramsay AJ, Husband AJ, Ramshaw IA et al. The role of Interleukin-6 in mucosal IgA antibody response in vivo. *Science* 1994; 264: 561-3.
23. Morrison DC, Ryan JL. Bacterial endotoxin and host immune responses. *Adv. Immunol.* 1979; 28: 293-450.
24. Cebra JJ, Shroff KE. Peyer's patches as inductive Sites for IgA Commitment. In: Ogra PL, Lamm ME, McGhee JR, (eds). Handbook of Mucosal Immunology. Academic Press Inc., 1994; 151-8.
25. Weisz-Carrington P, Roux ME, McWilliams M, Phillips-Quagliata JM and Lamm ME. Organ and isotype distribution of plasma cells producing specific antibody after oral immunization: Evidence for a generalized secretory immune system. *J Immunol* 1979; 123: 1705-8.
26. Cripps AW, Dunkley ML, Clancy RL, Wallace F, Buret A, Taylor DC. An animal model to study the mechanisms of respiratory tract by intestinal immunization. In: Mestecky J, Russell MW, Jackson S, Michalek SN, Tlaskalová-Hogenová H and Stezzl J. Advances in Mucosal Immunology. Part B. New York: Plenum Press, 1995, 749-53.
27. Clancy RL, Pang G, Cripps A. Mucosal responsiveness as a determinant of protection mechanisms induced by oral immunization. In: Mestecky J, Russell MW, Jackson S, Michalek SN, Tlaskalová-Hogenová H and Stezzl J. Advances in Mucosal Immunology. Part B. New York: Plenum Press, 1995; 1417-22.
28. Bene MC, Zanin C, Perrin P, Perruchet AM, Mole CM, Faure GC. Specific antibody-producing cells in humans after oral immunization with a ribosomal vaccine Ribomunyl™. In: Mestecky J, Russell MW, Jackson S, Michalek SN, Tlaskalová-Hogenová H and Stezzl J. Advances in Mucosal Immunology. Part B. New York: Plenum Press, 1995, 1563-6.