Immune dynamics of the digestive tract

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The digestive system in chickens undergoes dramatic transformation during the immediate post-hatch period in terms of bacterial colonization and the demand to digest and absorb complex adult-type food stuffs. These changes call for rapid adaptations of several physiological systems, including digestive capacity, barrier formation and development of protective immunity. Notwithstanding the significant protection provided by maternal antibodies (present in both serum and gut yolk), the chick must rapidly consolidate the structure and function of its own enteral immune system. We have investigated the development and maturation of the intestinal immune system in chickens in terms of the conditions required for development of adequate protective immunity. We show a unique biphasic pattern of lymphocyte colonization of the intestine and gradual maturation of T cell functions by two weeks post hatch. The first maturation phase of the adaptive GALT occurs during the first week of life and is characterized by establishment of lymphocytic populations in the intestine. The second phase of maturation which takes place during the second week of life is characterized by functional maturation which is demonstrated by increase in chIL2, chIFNγ and the ability to mount humoral response to enteric administered proteins. We tested the development of the innate GALT in newly hatched chicks. Polymorphonuclear cells increased in number during the first two weeks post-hatch resulting from both migration and in-situ development in extra-medullar foci. Expression of proinflammatory cytokine genes was basal at hatch but increased rapidly thereafter, so that by day 2, levels were similar to those observed on day 7. Expression of the IgA transporter gene in epithelial cells occurred post-hatch in correlation with development of adaptive immunity. The rapid increase in gene expression is related to the exposure to environmental microflora and demonstrates the capability of the enteric immune system to respond rapidly to inflammatory stimuli of external origin.

Keywords: GALT; immune response; chicken; digestive tract; immune development

Introduction

The structure and function of the digestive tract reflect the feeding habits of animals. Thus, the gut of predators is structured differently from that of herbivores and the digestive tracts of herbivores differ structurally depending on the site of cellulose digestion (1, 2). Another cause for difference in function between digestive systems relates to factors determining the development of digestive function in the gut. Thus, the rate of gut development, both anatomically and functionally, is different between species that immediately proceed to forage an adult type diet and those that are fed processed foods or milk by parents (1). Several bird species, including Gallus, proceed to forage immediately at hatch (3). This intake of an omnivorous diet requires the rapid adaptation of the digestive tract to accommodate breakdown and absorption of complex food stuffs (1, 4, 5). Concomitant with the exposure to an adult-type diet, the intestinal tract of these birds immediately becomes inhabited by microflora (6-10). Interestingly in Gallus sp., the major site for bacterial colonization is the large intestine, particularly the two cecal horns (2, 7, 9), and colonization occurs by rectal as well as by oral routes (7, 9, 10).

Consequent to the rapid colonization of the gut by commensal bacteria, as well as the possible entry of pathogenic bacteria, a parallel rapid development of gut associated lymphoid tissue (GALT) is
The objective of this paper is to describe the dynamics of immune system activity in the developing gut of the chick during the first two weeks post-hatch.

Materials and methods

The methodology used in these studies has been described in detail in several of our publications(11-15). The reader is referred to these manuscripts for further details. In brief, newly-hatched, unvaccinated Ross broiler chicks were used. Chicks were placed in floor pens on wood-shavings in isolated, disease-free, light and temperature controlled rooms at 32°C for the first week post-hatch followed by 28°C during the second week. Pathogen free feed and water were supplied ad libitum for the entire experimental period. Experimental procedures were approved by the Animal Care and Welfare Committee of our Institute.

Light microscopy
Tissue sections (2-3 μm) were scored (x 600 under immersion oil) for the presence of heterophils.

Immunizations
Rectal immunization was performed by gently placing a micropipette tip above the anal lips, and slowly dripping the solution into the cloacal opening, thus allowing the chick to voluntarily take up the solution via retrograde peristalsis. Oral immunization was performed by gently placing a blunt-tipped feeding needle above the tongue, and slowly dripping the solution into the pharynx, thus allowing the chick to voluntarily swallow the solution.

Antibody measurement
Antibody responses were measured in sera and media collected from cultured intestinal segments. Antigen specific antibody levels were determined by ELISA.

Flow Cytometric Analysis
Lymphocytes were analyzed by a FACScan equipped with CellQuest software (Becton Dickinson, Rutherford, NJ). Fluorescence data from at least 100,000 cells were collected from each sample.

Oligonucleotide Primer Pair Design
Specific oligonucleotide primer pairs were designed and synthesized according to published chicken sequences (GeneBank). Computer searches and sequence alignments were performed by means of software from Genetics Computer Group Inc. (Madison, WI, USA).

Gene expression assays
Total RNA was extracted from tissue slices using TRI reagent (Molecular Research Center, Cincinnati, OH, USA). Identical quantities of RNA were then reverse transcribed into cDNA, and the expression levels of various gene products were determined by a semi-quantitative PCR.

Statistics
Means of groups (n ≥6 chicks) were individually compared to that determined on the day of hatch. Significance of this comparison was determined by an unpaired t-test. T-test values of 0.005 – 0.05 were considered to be statistically significant, values of 0.05 – 0.1 were of marginal significance, and values larger than 0.1 were of no statistical significance.

Results and discussion
We initially followed the population of GALT by lymphocytes. The CD3γδ antigen is expressed on avian T lymphocytes and is involved in signal transduction pathways leading to T cell activation. Basal levels of CD3γδ mRNA were observed in all intestinal segments of newly hatched chicks. This
primary colonization remained almost unchanged during the first days post-hatch, while at 4 d post-hatch a dramatic increase in CD3+ cells was observed, which was significant at all sites examined (P<0.05). Further increases in CD3+ cell population occurred after day 4, however, these increases were of smaller magnitude. The dynamics of seeding patterns were similar in all intestinal segments tested except for the colon, in which after day 4 post-hatch, cell frequency was lowest in this segment. To study developmental and functional maturation of the intestinal T cell population, IL-2 mRNA levels were measured. Levels of IL-2 mRNA increased in the hind gut and in the ileum starting at day 4 post-hatch with a further major increase observed during the second week post-hatch. In the duodenum and jejunum there were little changes in IL-2 mRNA levels until day 8 post-hatch after which profound increases in IL-2 mRNA levels were observed. Correlations between the mRNA levels of CD3γδ and IL-2 were determined at the different intestinal sites and the degree of correlation for both genes was significant throughout the intestines. Thus the dynamics of IL-2 mRNA expression in the intestine correspond to a seeding pattern of T- lymphocytes. Moreover, the results suggest that although T lymphocytes colonize the various parts of the intestine at the same time the functional maturation of the intestine is not uniform and is biphasic. Expression of IFNγ mRNA levels was used as a measure of effector cell functionality along the intestine. IFNγ mRNA expression similar to that of IL-2 demonstrated a biphasic functional maturation. The relationship between IFNγ mRNA and CD3γδ mRNA levels was examined, and at all intestinal sites, with the exception of the colon, significant correlations were found (p<0.05). In the colon the relationship tended towards significance (P<0.1). In addition, IFNγ mRNA levels were also correlated, throughout the intestine, with IL-2 mRNA levels. The correlation was significant (p<0.05) with the exception of the caecal tonsils where the relationship tended towards significance (P<0.1). Hence, the dynamics of IFNγ mRNA levels corresponded with those of intestinal T-cell colonization.

mRNA expression of the Bu-1 antigen was used as a marker to follow gut colonization by B lymphocytes. Basal levels of Bu-1 mRNA were detected along the intestine of newly hatched chicks. At hatch, Bu-1 mRNA levels were higher in the caecal tonsils compared to other parts of the intestine. Increases in Bu-1 mRNA were observed from 4 d post-hatch in the small intestines. The primary colonization of the hindgut remained unchanged until day 6 post-hatch whereupon a marked increase in Bu-1 mRNA levels occurred. These results imply that small intestine colonization by B lymphocytes preceded that of the hindgut. Bu-1 mRNA levels remained relatively unchanged in all intestinal segments between 8 and 12 d post-hatch. These results suggest a steady flow of B lymphocytes to the intestine after day 6. It is interesting to note that despite the early seeding of B lymphocytes in the colon, their frequency in that segment was the lowest of all segments tested, similar to that observed for T lymphocytes. To test whether seeding patterns and lymphocyte cytokine gene expression were appropriate indicators of the physiological status of GALT in the young chick, we studied antibody specific responses to enterally administered antigens. While oral protein antigens induced potent systemic and gut-specific antibody responses in adult chickens, they did not induce responses in chickens orally immunized prior to 10 days of age. To exclude the possibility that lack of responses at young ages was due to antigen or the route of administration, chicks were also immunized rectally at different time points post-hatch and antibody levels were determined. Antibody responses began to appear only in chicks challenged between 8 to 12 d post-hatch. The first antibody response was observed in this group 5 days after the last rectal immunization. These results are in agreement with the colonization and cytokine gene expression data and demonstrate the gradual maturation of the broiler GALT during the first two weeks of life.

A link between development of adaptive and innate immune functions can be demonstrated via development of the IgA transport system. Expression of the poly-Ig IgA transporter was determined in intestinal epithelial cells with time before and after hatching. The results clearly show that expression of this Ig transporter was not-detectable in pre-hatch epithelium, and expression gradually increased with age. The rate of expression correlated with development of gut IgA expression.

As adaptive immunity in the chick GALT appeared to mature towards the second week of life, the issue of immune protection during the first week of life was raised. Immune protection could be provided during the first week of life by two non-mutually exclusive systems: maternal antibodies – active systemically and in the gut cavity, and innate effector mechanisms – active along all mucosal surface linings. Innate effector mechanisms function to eliminate potential infection by either blocking
the entry of microorganisms, or by preventing their spreading prior to induction of adaptive immunity. We hypothesized that in addition to protection provided by maternal antibodies, innate effector mechanisms are active along the hatchling's gut, and enable immune protection during the period required for the functional maturation of adaptive immunity.

To study the development of innate immune cells in the gut we determined the temporal expression of several genes along the gut of newly hatched chicks. The selected genes represent various activities and functions of both innate cells (macrophages and granulocytes) as well as enterocytes. The products of these genes are involved in proinflammatory immune responses (chIL-1β and chIL-8), immune cell recruitment and activation (K203, chIL-8 and chIL-1β) and heterophil bactericidal activity (Gallinacin1 and Gallinacin 2) (16-22). Basal mRNA levels of chIL-1β, chIL-8 and K203 were detected at hatch in all intestinal segments tested. While the levels of these cytokine mRNAs increased during the first week of life, the magnitude of increase varied between intestinal segments. ChIL-1β mRNA levels barely increased above the day 0 basal levels in colon and ceca, while in the duodenum they increased 2 fold during the experimental period (P<0.05). A 2 fold increase in chIL-8 mRNA levels was observed in the duodenum by the end of the first week of life (P<0.05 for day 7), and a much higher increase was observed in the hind gut at the same time (4 and 10 fold increase in colon and cecum respectively; P<0.05 for day 7 in colon and for days 4 and 7 in Cecum). A rapid increase in K203 mRNA levels was already observed on day 2 post hatch in the duodenum and colon (5 and 4 fold respectively; P < 0.05 for all days), while a only a two-fold increase in mRNA for this chemokine was observed in the ceca only by day 7 (P<0.05).

The temporal expression of the heterophil β-defensins, Gallinacin 1 and Gallinacin 2, was of a different pattern than that described for the pro-inflammatory cytokines. mRNA levels of the two β-defensins decreased relative to day of hatch throughout the first week of life (P < 0.05 for all days excepting Gal 1 on day 2 post-hatch). This pattern of expression was uniform throughout the intestine. As defensins are synthesized and stored in granules during heterophil maturation (17, 23), these data indicated the presence of immature heterophils in the gut at the time of hatch and that the functional development of granules content occurred within 2 d from hatch.

The gradual decrease in expression of genes coding for granule contents may indicate that immature avian heterophils migrate to the gut during the pre-hatch period and complete their maturation in situ within a brief period post-hatch. To assess the heterophil status in the intestinal wall of hatchlings, we performed a histological survey of heterophil colonies during the first 2 d post-hatch. Mature heterophils and granulocytes in general were very rare in the lamina propria at hatch, but became evident by 2 d of age. Interestingly, rare granulocytic foci containing dividing, young and mature forms of heterophils were readily detected in the connective tissue external to the muscularis propria on the day of hatch. The connective tissue, which appeared to be essentially mesenchymal at this age, harbored small colonies in which clear mitotic divisions could be seen. The connective tissue bordering the muscularis mucosa also contained heterophil colonies in which mitotic cells were common too. As the intestine developed, the mesenchymal connective tissue matured to loose irregular collagenic connective tissue, the heterophil colonies containing mitotic cells gradually disappeared, and mature heterophils dispersed in the lamina propria. The number of mature heterophils increased with age in all intestinal segments examined, with the largest increase occurring in the cecum, where high numbers of cells were scored by d 7 of age. The histological data implies that the pre- and immediately post-hatch gut contained heterophils that retained their capacity to divide outside the bone marrow. This, together with above observations on β-defensin gene expression, indicate the gut to be a site in which granulocytes attain and complete their functional maturation.

To conclude, our data demonstrate two circumstances of innate preparedness in the developing gut. The first is independent of intestinal exposure to feed and bacteria, and is represented by a local extramedullary granulopoietic process that is supported by histological as well as by gene (PS1 and β-defensin) expression. This extramedullary development occurs close to hatch in the small gut, and was not observed in the large gut. The second circumstance, appears to depend upon exposure to feed and flora, and is represented by innate pro-inflammatory cytokine and chemokine gene expression. This response develops in the intestine of newly hatched chicks immediately after hatch, and is speculated to be linked with bacterial colonization of the intestine; thus, the functions are enhanced in the large gut which is the main site for bacterial colonization in the chick. Consequently, the newly hatched intestine
(epithelium as well as resident leukocytes) triggers, via pro-inflammatory mediators, the recruitment of blood-borne leukocytes, first innate cells and later on lymphocytes, to the developing intestine.

References

