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Effects of microbial stimulation on the number, size and activity of bronchus-associated lymphoid tissue (BALT) structures in the pig

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Summary. The development of bronchus-associated lymphoid tissue (BALT) was investigated in the pig, which is a species in which BALT is not found constantly. Different routes of contact with a specifically lung-pathogen bacterium *Actinobacillus (Haemophilus) pleuropneumoniae* were tested. Pigs, selected by bacteriological screening methods and the number of granulocytes in the bronchoalveolar lavage (BAL), were infected by aerosol. They were compared to previously enterally immunized pigs using active and inactivated bacteria. The development of BALT after the infection was compared to that in pigs with a single enteral, or no, contact with the bacterium. BALT was less frequent in these groups than in the infected pigs. Previously immunized pigs developed the highest number and the largest BALT with the most prominent morphological signs of activation. Immunization with living or inactivated bacteria did not cause histological differences.

BALT was preferentially located around bronchioli and small bronchi. Additional BALT predominantly occurred in the walls of larger bronchi. Definite compartments of T and B lymphocytes were not found in immunohistological studies of BALT. It was concluded that the development of BALT can be induced by different modes of microbial stimulation.

Keywords: bronchus-associated lymphoid tissue (BALT), pig, histology, mucosal immunity, *Actinobacillus (Haemophilus) pleuropneumoniae*

The respiratory system is a huge area exposed to a large number of different antigens, toxins, bacteria and viruses. Non-specific and specific mucosal defence mechanisms protect the individual against disease. The immune function of the lung is secured by lymphoid cells spread over different compartments (reviewed in Stein-Streilein 1988; Berman *et al.* 1990; Pabst 1990; Stein-Streilein & Toews 1990): (1) intravascular, (2) interstitial, (3) intra-alveolar and (4) in an organized form which was first described and named bronchus-associated lymphoid tissue (BALT) by Bienenstock *et al.* (1973a, b). Characteristically solitary follicle-like structures in the lamina propria of bronchi are covered by a flattened non-ciliated epithelium which is infiltrated by

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lymphocytes (Bienenstock & Johnston 1976). The number of goblet cells and the expression of the secretory component for the transepithelial transport of IgA is reduced (Gehrke & Pabst 1990). It has been postulated that BALT plays an important role in antigen uptake, antigen presentation and initiation of immune responses.

However, BALT is not a constitutive structure in all species. Its presence and number vary considerably between different species, ranging from 100% in rabbits and rats, 30–50% in pigs and mice, to its absence in cats and man (Pabst & Gehrke 1990).

Differences in the number and size of BALT structures in germ-free and conventional animals point to a development dependent on microbial antigen (Gregson *et al.* 1979a, b; Pabst & Gehrke 1990). Immunohistological studies on typical T- and B-cell areas in mammalian BALT produced conflicting results. Rabbits (Racz *et al.* 1977), rats (Van der Brugge-Gamelkoorn & Kraal 1985; Van der Brugge-Gamelkoorn & Sminia 1985; Simecka *et al.* 1986) and guinea-pigs (Van der Brugge-Gamelkoorn & Kraal 1985) have been investigated, but no data are yet available in pigs.

The present study addresses the following questions: (1) Can the development of BALT be induced in a species, in which under normal circumstances BALT is not always present, using one definite lung-pathogen bacterium? (2) Is there a difference whether antigen contact is performed once or twice, or using viable and inactivated bacteria? (3) Where does BALT predominantly develop and what is its histological structure? (4) Can compartmentalization in T- and B-cell regions be induced by controlled microbial stimulation?

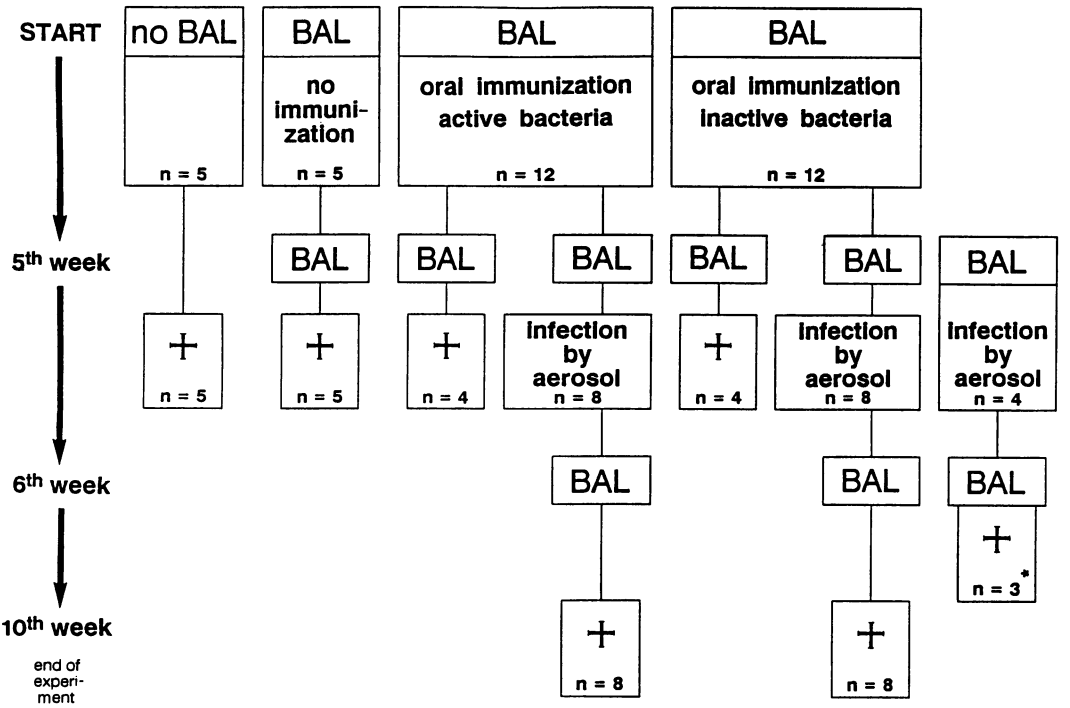
Methods

The lungs of pigs used in experiments to evaluate vaccination effects by aerosol challenge were made available for this histological study (K. Petzoldt & A. Hensel, to be published).

Male, castrated, 4-month-old German Landrace pigs ($n=38$) from a specific pathogen-free (SPF) breeding farm were used. Pigs not lavaged ($n=5$) were used as a control group. Before the experiment started bronchoalveolar lavage (BAL) was performed in the other 33 pigs. BAL was examined by bacteriological methods. The cellular composition of BAL was checked in Giemsa stained cytospin preparations. Only pigs with no evidence of lung-pathogen bacteria and less than 10% granulocytes in BAL were included in this study. The time schedule and design of our study is summarized in Table 1. To test the effect of BAL, pigs ($n=5$) were lavaged but not immunized. Another group ($n=24$) was immunized by swallowing lyophilized encapsulated bacteria *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* at a dose of 10^{11} bacteria. Active bacteria ($n=12$) and bacteria inactivated by radiation ($n=12$) were compared. The technique of immunization and preparation of the bacteria will be published separately. After the initial BAL, one group ($n=4$) was infected with the bacterium by aerosol at a dose of 10^9 viable bacteria, which corresponds to the LD_{50}, and the animals were sacrificed one week later. The other immunized animals ($n=16$) were challenged by aerosols containing 10^9 viable *Actinobacillus* (*Haemophilus*) *pleuropneumoniae*. The pigs were sacrificed at the times indicated in Table 1.

Immediately after sacrifice the lungs were removed. From each pig at least six samples of the right and left lung were taken from upper, basal, peripheral, central, ventral and dorsal areas, whereby only areas with no macroscopically identifiable pathologic changes were chosen. Areas which were macroscopically normal also showed no signs of pneumonia in histology. The microbiological data from these lungs and a detailed description of different parts of the lungs will be given in a separate paper. Tissue blocks were fixed in Schaffer's solution, embedded in glycolmethacrylate, cut at 2 μm and stained with Giemsa solution. The area of each section was measured and

Table 1. Time schedule and design of the present study.



* One pig died before the end of the study.

evaluated microscopically at a magnification of × 75. The extent and localization of BALT were registered. The extent of BALT per cm^2 of the embedded tissue was calculated.

To evaluate the localization of BALT, bronchi and bronchioli with and without BALT were counted in infected ($n=3$) as well as in immunized and infected pigs ($n=4$). Bronchi with cartilages were registered separately. An area of more than 21 cm^2 with about 900 airways was examined in each group.

Additionally four samples from each side of the lungs were frozen in liquid nitrogen. Cryostat sections were cut at 5 μm and fixed in methanol–acetone for 10 min at 4°C. Surface antigen was demonstrated by the alkaline phosphatase–anti-alkaline phosphatase (APAAP) method. The following antibodies were used: anti-CD2 (Mac 80, monoclonal antibody rat anti-pig; 1:300), kindly donated by Dr R.M. Binns, Cambridge, GB. The secondary antibody rabbit anti-mouse (Z 259, 1:50) and monoclonal APAAP-mouse (D 651, 1:50) were purchased from Dako, Hamburg, FRG. The distribution of T-cells was studied in 43 BALT taken from all animals orally immunized and infected. BALT was cross-sectioned at the largest diameter. The pattern of distribution was as follows: (1) diffuse, (2) more T-cells in the periphery than the centre, (3) more T-cells in the basal area than the area near to bronchial mucosa, (4) more T-cells near to bronchial mucosa than basally. BALT composed of more than one follicle-like structure was registered separately.

For statistical analysis the mean, standard

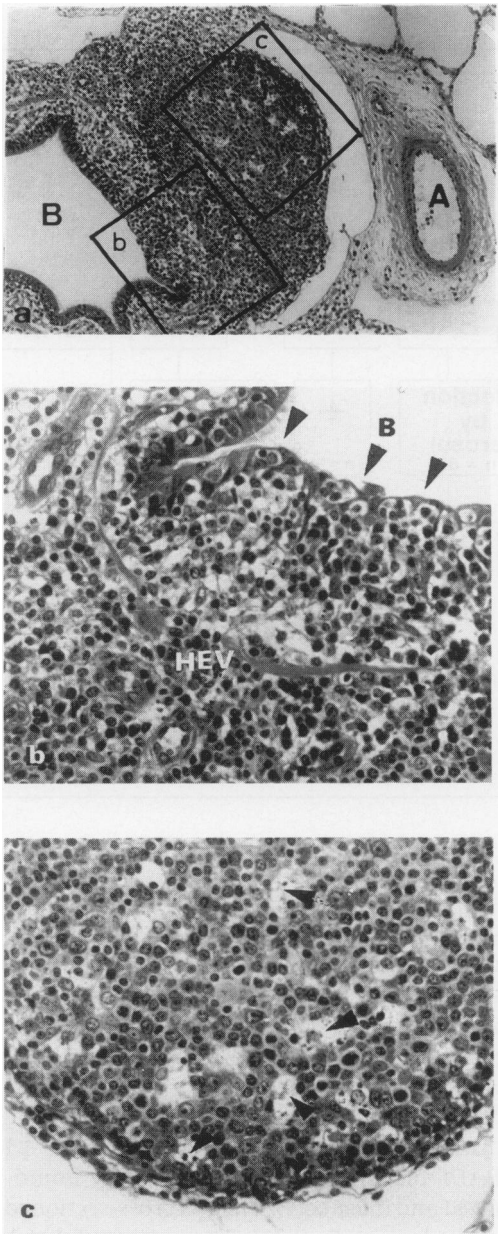


Fig. 1. a, Histological section of BALT in a pig which had been exposed to *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* by aerosol after previous enteral immunization. ×75. b, Prominent lymphocytic infiltration of the overlying epithelium (arrows) and typical high endothelial venules (HEV). c, Numerous macrophages full of nuclear cell debris (arrows). ×245. Plastic embedding, Giemsa, 2 μm. B, Bronchus; A, artery.

deviation and standard error were calculated. Differences between groups were evaluated by the non-parametric test of Mann and Whitney.

Results

In all pigs infected with *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* by aerosol a significantly higher number ($P<0{\cdot}001$) of BALT structures were found compared to the different control groups without infection (Table 2). The four control groups were not statistically different concerning the number of BALT structures. Previously orally immunized animals produced significantly more BALT than pigs without immunization ($P<0{\cdot}01$). Using viable or inactivated bacteria did not influence the number of BALT structures ($P>0{\cdot}05$).

In immunized and subsequently infected animals the amount of BALT was on average greater (Fig. 1) than in the infected animals and in the non-infected controls (Fig. 2). BALT of previously immunized pigs was often characterized by larger lymphoid cells and mitoses in the centre and smaller lymphocytes in the periphery. Frequently, macrophages full of nuclear debris and typical high endothelial venules (HEV) were disseminated in the lymphoid aggregates. A prominent lymphocytic infiltration of the overlying epithelium was often present (Fig. 1). At the base of the BALT an endothel-lined cleft was found with some lymphocytes inside probably an efferent lymphatic vessel.

In the infected animals 97% of the BALT was associated with bronchioli and 3% with bronchi; in immunized and infected animals, 83·2 and 16·8% respectively. The incidence of BALT increased from 3·5% after a single antigen contact to the respiratory mucosa to 13·2% after a second exposure. Previous antigen challenge increased the number of BALT structures associated with bronchioli by a factor of 3·8 ($P<0{\cdot}001$) and with bronchi by about 14 times ($P<0{\cdot}001$).

Immunohistological investigation of sections with BALT taken from the orally

Table 2. BALT in different microbial situations

Microbial environment	Age (months)	*n*	Mean evaluated area per animal (cm^2)	Mean number of BALT structures per cm^2	Animals with BALT (%)
Infected with *Act. pleurop.* by aerosol	4	20			
no pretreatment	4	*4	7.4±1.2	1.7±0.4	100
orally immunized with viable bacteria	4	8	5.6±1.6	7.8±4.4	100
orally immunized with inactive bacteria	4	8	6.9±1.6	5.4±1.3	100
Control groups: no infection	4	18			
orally immunized with viable bacteria	4	4	9.9±0.5	0.8±0.4	100
orally immunized with inactive bacteria	4	4	9.3±0.8	0.5±0.8	100
only BAL	4	5	9.6±1.4	0.7±0.6	100
no treatment at all, no BAL	4	5	8.3±0.8	0.3±0.4	80

*One pig died before the end of the study.

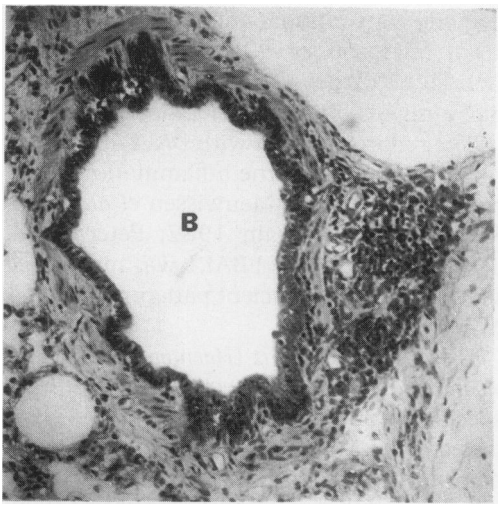


Fig. 2. Histologic section of BALT in conventional pig. Plastic embedding, Giemsa, 2 μm. ×135. B, Bronchus.

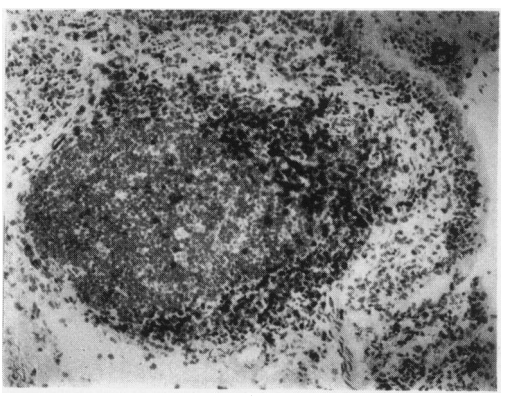


Fig. 3. Immunohistology of BALT. Distribution of T lymphocytes using an anti-CD2 monoclonal antibody (Mac 80). Cryostat section, alkaline phosphatase–anti alkaline phosphatase (APAAP) method. ×90. Bronchus.

immunized and infected animals showed a diffuse distribution of T-cells in 60% of the cases. More T-cells were detected in the periphery than in the centre in 30%. Predominance of T-cells at the base of the mucosa-associated area of BALT occurred in about 10% (Fig. 3). BALT consisted of two or three follicle-like structures in 5%.

Discussion

The role of BALT in the immune function of the lung is still under discussion. Major species differences have been found concerning the presence of BALT (Pabst & Gehrke 1990). Differences in the presence and number of BALT structures between germ-

free and conventional animals (Jericho *et al.* 1971; Gregson *et al.* 1979a, b; Pabst & Gehrke 1990) point to an influence of microbial antigens on the development of BALT.

The reports on man with BALT-like structures mostly record the inflammation of the respiratory system (Meuwissen *et al.* 1975; Meuwissen & Hussain 1982; Peters *et al.* 1990). Hyperplasia of BALT was mentioned in a few immunodeficient patients (Kradin & Mark 1983).

Using *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* for the infection in swine, a specifically lung-pathogen bacterium was tested comparable to human-pathogen *Haemophilus influenzae* (Kilian *et al.* 1979). The infection of the animals was performed by defined nose-only aerosol exposure in order to imitate the usual entrance of bacteria to the lung. For the immunization procedure direct stimulation of the respiratory mucosa was avoided, so changes in the lung could be studied according to whether the antigen exposure was performed once or twice. Histological changes in BALT and its lymphoepithelium in the present experiment were similar to those previously described for rats after antigenic challenge (Gregson *et al.* 1979b; Van der Brugge-Gamelkoorn *et al.* 1986).

Previous studies mainly investigated macroscopically detectable BALT (Bienenstock *et al.* 1973a; Plesch 1982; Sminia *et al.* 1989). It was often found in the wall of larger bronchi near to bifurcations. In the few reports on humans (Bienenstock *et al.* 1973a; Meuwissen *et al.* 1975; Meuwissen & Hussain 1982) BALT was mentioned to be more conspicuous around bronchioli and smaller bronchi. In the present study the majority of the lymphoid aggregates were also found associated to bronchioli and smaller bronchi without cartilage. These data agree with the results of a recent morphologic study on the intrapulmonary airways in swine (Huang *et al.* 1990). Thus, the pig appears to be an experimental animal more comparable to man than the frequently used rodents. Surprisingly the increase in BALT was not equally distributed among the airways. Bronchi with cartilage were significantly more likely to develop additional BALT after repetitive antigen exposure.

Separate compartments with distinct T- and B-cell areas were described in the BALT of rabbits (Racz *et al.* 1977) and chickens (Sminia *et al.* 1989). In rats the situation remains controversial (Van der Brugge-Gamelkoorn & Kraal 1985; Van der Brugge-Gamelkoorn & Sminia 1985; Simecka *et al.* 1986). After microbial stimulation compartmentalization occurred in some pigs, but was not a constant finding. Further studies might show that there is a typical sequence of histotopographical localization of lymphocyte subsets, and more antibodies should be tested when they become available for pigs, e.g. adhesion molecules, memory cells or activated lymphocytes.

It is possible to conclude from our studies that the generation of BALT can be induced in a species in which BALT is not a constant finding. The number and morphology of BALT structures can be manipulated by different modes of microbial stimulation using one lung-pathogen bacterium. The capacity to influence the development of BALT should be tested with different microbial and other antigens. It should be investigated whether BALT is the morphological response to a specific antigen or whether it is a more general reaction of the lung.

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