Clin. exp. Immunol. (1992) **90**, 223–227

Cellular changes in the bronchoalveolar lavage (BAL) of pigs, following immunization by the enteral or respiratory route

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(Accepted for publication 22 May 1992)

SUMMARY

Normal young pigs were immunized by the oral or aerogenic route with the viable or inactivated lung-pathogenic bacterium *Actinobacillus (Haemophilus) pleuropneumoniae*. Three weeks later the cellular composition as well as the lymphocyte subset composition of the bronchoalveolar space were examined by BAL. Lymphocytes in the lavage increased significantly, including $CD4^+$ and $CD8^+$ T cells. After oral immunization a dramatic increase of plasma cells and lymphoid blasts was found. Among immunoglobulin-positive lymphocytes IgG^+ cells showed the most pronounced increase. For most lymphocyte subsets there was no difference between viable and inactivated bacteria. Oral immunization with a lung-pathogenic bacterium results in increased numbers of lymphocytes in the bronchoalveolar space and might play a critical role in protection against lower respiratory tract infections.

Keywords bronchoalveolar lavage immunization *Actinobacillus (Haemophilus) pleuropneumoniae* pig

INTRODUCTION

After the gastrointestinal tract the respiratory tract is the largest mucous surface continuously exposed to tremendous numbers and types of microbes and toxic agents from the environment. Acute, recurrent and chronic respiratory tract infections are of great clinical relevance in medicine and veterinary practice. There are humoral and cellular mechanisms at work protecting the respiratory tract, with the IgA system playing a predominant role for the upper and IgG for the lower respiratory tract (for review see [1,2]). The mucosal immune system of the respiratory tract is integrated in the common mucosal-associated lymphoid tissue (MALT) by the traffic of lymphoid cells between these sites (for reviews see [3,4]). These studies on animals and man have focused mainly on the IgA system and on cells in the lamina propria of the bronchial tract. Based on the MALT concept, oral immunizations with lung-pathogenic bacteria, e.g. non-typable *Haemophilus influenzae*, have significantly reduced the incidence of acute bronchitis [5]. In addition, oral immunization decreased the colonization of the oropharynx with the respective bacteria or virus as demonstrated in experimental animals and man [6–9]. The lymphoid cells in the bronchoalveolar lumen, however, have not been studied in this respect. These cells can easily be obtained by BAL, a standardized technique which has been recommended as an important diagnostic procedure for several lung diseases [10,11]. BAL has also been performed in experimental animals such as the pig, to study the effect of different routes of immunization [12]. Immunization by aerosol is of special veterinary interest for practical and economic reasons [13].

The aim of the present study was two-fold: to compare the influence of (i) oral with aerogenic immunization, and (ii) live with inactivated bacteria on the number and subset composition of lymphocytes in the bronchoalveolar space. Young pigs were used for this study. The bacterium *Actinobacillus pleuropneumoniae* (A.p.) was chosen because it is not only of great clinical veterinary relevance in young pigs [14], but also comparable to *H. influenzae* in many respects [15,16].

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MATERIALS AND METHODS

In this study 37 male, castrated, 4-month-old German landrace pigs were used from a specific pathogen-free (SPF) breeding farm. The design of this study is depicted in Fig. 1. Before the experiment BAL had been performed in all pigs, as part of the selection procedure. Only pigs with no evidence of lung-pathogenic bacteria and less than 10% granulocytes in BAL were chosen for this study. The control group ($n=5$) was not immunized. Within a week after BAL the pigs were immunized with A.p. One group ($n=16$) swallowed lyophilized encapsulated A.p. bacteria at a dose of 10^{11} bacteria. Eight of these animals received viable and the other eight inactive bacteria. A second group ($n=16$) was exposed to an aerosol containing 10^7

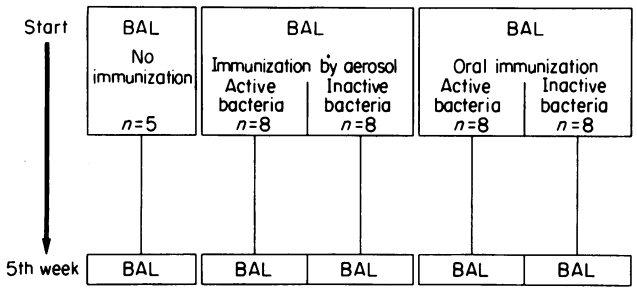

Fig. 1. Design of the present study.

bacteria, either in viable form ($n=8$) or inactivated ($n=8$), in a special aerosol chamber. The techniques of immunization and preparation of the bacteria will be published in a separate paper. Using the enteral and the respiratory route for immunization, viable bacteria were compared with bacteria previously inactivated by radiation with 500 Gy. After 3 weeks (i.e. in the fifth week after the initial lavage) BAL was again performed in all groups (Fig. 1).

A modified method of BAL [17] was performed in anaesthetized pigs using a flexible fibre optic bronchoscope. A dose of 50 ml PBS was instilled in aliquots of 10 ml into the right main lobe. Nucleated cells and erythrocytes were counted in a Neubauer chamber using phase contrast microscopy. Total numbers were calculated. Cytospins were prepared (200 *g*, 3 min), air-dried, stained with Giemsa solution or frozen at −20°C. BAL cells were classified on these Giemsa-stained slides as macrophages, granulocytes, lymphocytes, plasma cells and blasts, with at least 500 cells being differentiated.

Lymphocyte subsets were identified by MoAbs using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method. The following MoAbs were used: Mac 80 (anti-CD2) kindly donated by Dr R. M. Binns (Cambridge, UK), SL2 (anti-CD8), PT4 (anti-CD4) kindly donated as ascites by A. Saalmüller (Tübingen, Germany). The origin and specificity of these MoAbs has recently been reviewed [18]. Immunoglobulin-positive lymphocytes were detected by MoAbs against pig IgA, IgG, IgM, kindly donated by Dr A. Bianchi (Lelystad, The Netherlands). The second antibody rabbit anti-mouse (Z259, 1:50) and the monoclonal APAAP-mouse (D651, 1:50) were purchased from Dako (Hamburg, Germany). By testing serial dilutions of antibodies and comparing the percentages of alveolar macrophages stained by a MoAb, immunoglobulin-positive lymphocytes could be differentiated from lightly positive macrophages with adherent immunoglobulins. At least 500 cells were counted to evaluate the percentage of cells positive for T cell markers. At least 1000 cells were evaluated for the immunoglobulin-positive subsets at a magnification of ×63. The total number of cells was calculated.

Statistical analysis

Each variable was tested as to whether the values were distributed normally. The mean, s.d. and s.e.m. were calculated. Significance was tested by Student's *t*-test or by the non-parametric test of Wilcoxon. Statistical differences were taken as significant with $P<0{\cdot}05$, if not otherwise stated.

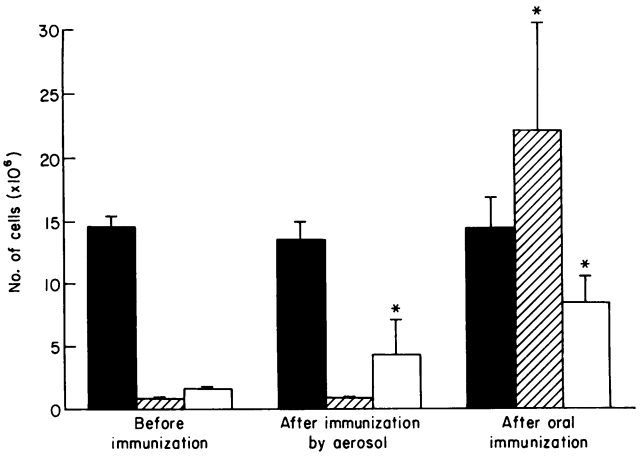

Fig. 2. Total number of cells in BAL before and after oral and respiratory immunization. Macrophages (■), granulocytes (PMN, ▩) and lymphocytes (□) are shown. Mean ± s.e.m. * Significant differences from the preimmunization values.

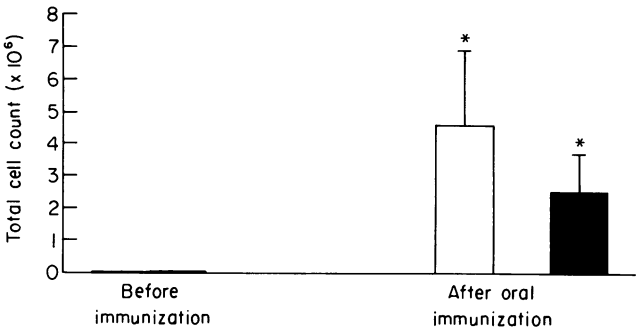

Fig. 3. Total cell count of plasma cells (□) and blasts (■) identified in Giemsa-stained cytospin preparations of BAL after oral immunization with *Actinobacillus (Haemophilus) pleuropneumoniae*. Mean ± s.e.m. * Significant differences from preimmunization data.

RESULTS

In the control group the cell number, the cellular composition in Giemsa-stained cytospots and the T and B lymphocyte subsets were comparable to the preimmunization data of the other groups. When the data of the initial and the second BAL were compared (data not shown), no significant differences were obtained. Thus, the BAL itself did not affect lymphocytes in the bronchoalveolar space. This was not so for the immunized pigs. As no significant differences were seen between the pigs immunized with viable and those with inactive bacteria, the data were pooled for most parameters, but detailed data are given for immunoglobulin-positive lymphocytes. The total number of macrophages remained constant before and after immunization by the oral or aerosol route. The total number of granulocytes, however, was significantly higher in the orally immunized animals ($P<0{\cdot}05$). This effect was not seen using the respiratory route (Fig. 2). The total number of lymphocytes increased in both groups after immunization ($P<0{\cdot}01$).

In the BAL of normal pigs plasma cells are extremely rare (< 0·1%) and blasts are not normally found when only 1000 cells

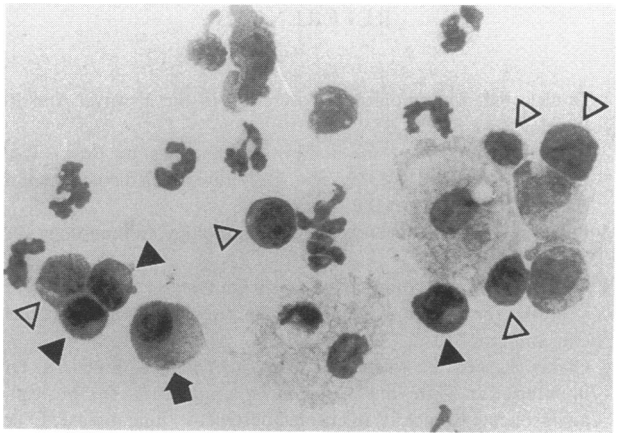

Fig. 4. Occurrence of lymphocytes (△), plasma cells (▲) and blasts (arrow) in BAL after enteral immunization. Giemsa-stained cytospin preparation, about × 1000.

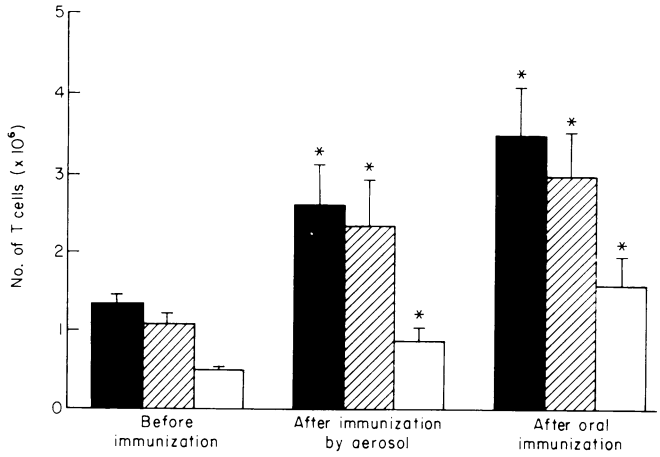

Fig. 5. Subsets of T lymphocytes in BAL before and after enteral and respiratory immunization. MoAbs used were: Mac80 (anti-CD2), SL2 (anti-CD8), PT4 (anti-CD4). Mean ± s.e.m. * Significant differences from preimmunization data. ■, CD2; ▨, CD8; □, CD4.

are differentiated. After oral immunization with A.p., however, the percentage of plasma cells increased significantly ($P < 0{\cdot}01$) to a mean of 4·2% and a mean of 2·4% lymphoid blast cells were found (Fig. 3). A typical cytospot of BAL after enteral immunization is shown in Fig. 4. A tendency to a higher number of plasma cells was also found after immunization by aerosol, although these changes were not statistically significant.

Most lymphocytes found in the initial BAL were $CD2^+$ T lymphocytes. The ratio between $CD2^+$ cells and the sum of immunoglobulin-positive cells ($IgG^+ + IgM^+ + IgA^+$) was about 10:1. The mean ratio between $CD4^+$ and $CD8^+$ cells was 0·46.

The increase in the total number of lymphocytes, mainly due to immunization, was caused by an increase in T cells (Fig. 5). The ratio of CD4/CD8 cells remained constant after immunization by both routes. Thus, all lymphocyte subsets increased after immunization by both routes.

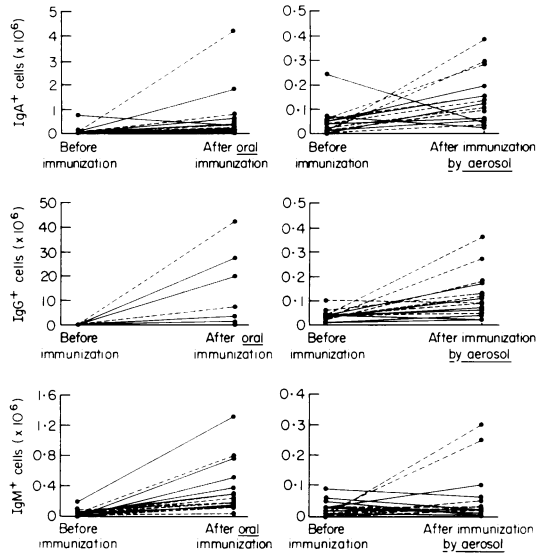

Fig. 6. Total number of immunoglobulin-positive cells in BAL. ——, Viable bacteria; · · · ·, inactive bacteria.

With regard to the sum of all immunoglobulin-positive cells, an increase was observed in all immunized pigs ($P < 0{\cdot}01$). Different patterns were obvious for IgA^+, IgG^+ and IgM^+ cells. IgA^+ cells increased after immunization with inactivated bacteria ($P < 0{\cdot}01$ using the respiratory route; $P < 0{\cdot}05$ using the enteral route). After immunization by aerosol IgG^+ cells increased by a factor of about 3, in enterally immunized pigs by a factor of about 125. IgM^+ cells increased after oral application ($P < 0{\cdot}01$) using viable as well as inactivated bacteria. For IgM^+ lymphocytes only the group immunized with inactivated bacteria via the aerosol route produced a statistically significant increase ($P < 0{\cdot}05$) (Fig. 6).

DISCUSSION

Oral immunization against lung-pathogenic bacteria has been shown to be effective in clinical studies, resulting in a 10-fold reduction of the incidence of infection [5]. However, there was no clear correlation between clinical protection and presence of either *H. influenzae* or antibodies to this bacterium in the saliva. Thus, the mechanisms of the enteric-bronchial route are still under debate.

The pigs in this study were later infected with *A. pleuropneumoniae* and were clinically protected from respiratory disease or death, while control animals succumbed. These data will be published separately. The present part of this study revealed several interesting findings. The increase of granulocytes in BAL after oral immunization is difficult to explain. It cannot be the effect of the initial BAL, as this increase was not seen in the control pigs. It has recently been shown in man that the appearance of granulocytes after BAL is only transient and disappears after approximately 3 days [19]. All microbiological efforts to find mycoplasms or typical lung-pathogenic viral infections in these pigs failed (data not shown). The reason for

the granulocytes in the bronchoalveolar space after oral immunization remains obscure. There was no clinical effect on the behaviour and health of the pigs.

The present data are consistent with the hypothesis that lymphocytes not only migrate from the gut to the lamina propria of the bronchi and trachea [20,21], but also into the bronchoalveolar lumen. In previous studies the migratory route of IgA-producing cells was mainly followed. The pronounced increase of IgG^+ lymphocytes in BAL is consistent with the topographical differences in immunoglobulin content, with more IgA in the upper and IgG in the lower respiratory tract [12]. Obviously the lavage mainly represents smaller bronchi and the alveolar space. In a recent study in dogs, lavaged cells from an immunized lung lobe produced high levels of antibodies even a few years after immunization [22]. Thus, long-term there are localized antibody-producing cells in the bronchoalveolar space. The occurrence of plasma cells and lymphoid blasts in BAL might be surprising at first sight, as these cells are not normally present. However, after local immunization plasma cells have been documented in the lung interstitium and in the alveoli [23]. The present data might indicate an emigration of plasma cells from the lung interstitium into bronchoalveolar space and these might contribute to the antibodies found in the BAL fluid. Future studies should clarify whether the B lymphocytes and plasma cells express and secrete specific antibodies.

The factors which regulate the migration of lymphocytes from the lung interstitium to the airspace are as yet unknown [24,25]. The common mucosal immune system is often seen as being linked together only by the traffic of plasma cell precursors [3,4]. In the present study, small T lymphocytes, including $CD4^+$ and $CD8^+$ cells, were significantly increased in BAL at 3 weeks after enteric immunization. These results are consistent with recent findings in rats. When thoracic duct T lymphocytes were transferred from rats immunized by intra-Peyer's patch injection to naive animals, the recipients were able to clear nontypable *H. influenzae* from the respiratory tract better than control animals [26]. Thus, antigen-primed T cells derived from the gut-associated lymphoid tissue might be more important than specific antibodies in the respiratory tract. There is growing evidence that T lymphocytes from the bronchoalveolar space differ from lymphocytes in the blood, e.g. expressing the IL-2-receptor more frequently, the homing receptor Leu-8 less often, representing memory T cell type receptors and typical adhesion molecules like VLA-4 more often (for review see [27]). Immune reactions in the lung include alterations in the B and T lymphocyte subsets.

The differences between the pigs immunized by the oral and the aerosol route could partly be due to different amounts of bacteria used. A combination of the oral with the respiratory route of immunization seems to be the best way of protecting the respiratory tract, as shown in mice [6] and rats [28]. The recent advances in targeting antigen for an effective induction of secretory immune response in the gut might also be important in immunizing the respiratory tract [29].

ACKNOWLEDGMENTS

This study was supported by a grant from the German Federal Ministry of Research and Technology (01 KE 88141) and from the Fraunhofer-Gesellschaft, Munich, Germany. The technical assistance of M. Peter, H. Riechers and A. Skupin and the correction of the English by S. Fryk are gratefully acknowledged.

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