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# Primary Antibody Responses to Thymus-Independent Antigens in the Lungs and Hilar Lymph Nodes of Mice

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**B lymphocytes from the pulmonary lymphoid tissues were stimulated with a variety of thymus-independent (TI) antigens by intratracheal (i.t.) immunization. Immune responses in the lungs and hilar lymph nodes (HLN), which are part of the localized lymphoid tissue, as well as in the spleen, the systemic lymphoid organ, were studied. Thus, primary i.t. immunization of mice with the TI-1 antigen trinitrophenyl-lipopolysaccharide (TNP-LPS) elicited both antigen-specific and polyclonal plaque-forming cell responses from HLN, lung, and splenic B lymphocytes. These responses appeared as early as 3 days after immunization and declined by day 7. Similar immunization with another TI-1 antigen, TNP-*Brucella abortus*, resulted in anti-TNP responses in both pulmonary and systemic lymphoid tissues, although the kinetics of the antibody response were different than those to TNP-LPS. Interestingly an i.t. immunization with a TI-2 antigen, TNP-Ficoll, failed to induce an anti-TNP PFC response from HLN and lung B cells, although there was good antibody formation from splenic B cells. Antibody response to TNP-Ficoll was restored in pulmonary tissues when mice were immunized with TNP-Ficoll mixed with unconjugated *B. abortus*. In conclusion, our results indicate that TI-1 and TI-2 antigens differ in their ability to induce antibody responses in the pulmonary lymphoid tissues. The inability of TNP-Ficoll to elicit an antibody response in pulmonary lymphoid tissues has significance in the development of vaccines containing bacterial polysaccharides.**

The lung is a principal biological link between the body and the outside environment, with a surface area about 30 times that of skin (41). The lungs and upper respiratory tract constitute an important natural route for the entry of antigens and potential pathogens into the body. The pulmonary lymphoid system consists of a specialized lymphoepithelium called bronchus-associated lymphoid tissue, lymphoreticular aggregates and alveolar macrophages in the lungs and the hilar lymph nodes (HLN) situated on either side of the trachea near the bronchus (18).

Despite the importance of lung-associated immune tissues in resistance to infection (1, 5, 8, 15, 34, 38), little information is available on how different effector functions are carried out by these cells and on the relationship between the pulmonary and systemic immune responses. Studies by other investigators to elucidate the mechanism of the appearance of antibody responses in the pulmonary tissues of mice and dogs have employed sheep erythrocytes (SRBC), which belong to the category of thymus-dependent antigens (4, 10, 20, 22, 39). Since many of the microbial organisms contain thymus-independent (TI) antigens (11, 27, 30, 43), we felt it was important to analyze the capacity of the pulmonary immune system to respond to these antigens. TI antigens are classified as TI-1 and TI-2 based on their ability to induce antibody formation in immune defective CBA/N mice and in neonatal mice (6, 24, 28, 29). The TI-1 antigens employed in this study were trinitrophenyl-lipopolysaccharide (TNP-LPS) and TNP-*Brucella abortus* (TNP-BA). In the TI-2 antigen category, TNP-Ficoll was tested.

Recently, we have reported that TNP-Ficoll is unable to induce antibody formation in the popliteal and inguinal lymph nodes after subcutaneous immunization in the footpads but elicited a good anti-TNP response from splenic B cells of the same mice (12). Further, this dichotomy between the induction of lymph node and splenic B cell responses was shown to be a general property of a variety of TI-2 antigens (S. N. Goud, A. M. Kaplan, and B. Subbarao, Reg. Immunol., in press). Interestingly, the mesenteric lymph node B cells, which belong to the category of gut-associated lymphoid tissue, responded well to TNP-Ficoll. Therefore, we wanted to determine if TNP-Ficoll could induce antibody-forming cells in lung and HLN B cells after intratracheal (i.t.) immunization. In contrast to TNP-Ficoll, TNP-BA has been shown to induce good plaque-forming cell (PFC) responses in both popliteal and inguinal lymph nodes as well as in the spleen after subcutaneous immunization (12). Hence, in the present investigation, we tested the ability of TNP-BA to induce antibody formation in pulmonary lymphoid tissues. TNP-LPS was also tested because LPS is present in most of the gram-negative bacteria which cause respiratory distress syndrome in humans (35).

The results of this investigation show that a primary antibody response in lung and HLN B lymphocytes was obtained sooner with TNP-LPS than with TNP-BA. A polyclonal antibody response was observed with TNP-LPS in both lung and HLN B cells. Immunization with the TI-2 antigen TNP-Ficoll failed to elicit an antibody response in the lungs and HLN, although a good response was obtained in the spleens of these animals. Antibody response to TNP-Ficoll was restored in pulmonary lymphoid tissues when mice were immunized with TNP-Ficoll mixed with *B. abortus*.

## MATERIALS AND METHODS

**Animals.** Ten- to twelve-week-old DBA/2J female mice obtained from Jackson Laboratory, Bar Harbor, Maine, were used in this study. They were kept in microisolator cages (Lab Products Inc., Maywood, N.J.). These cages were housed in Bioclean Chambers (Hazleton Systems, Inc. Aberdeen, Md.). The animals had free access to food (Purina

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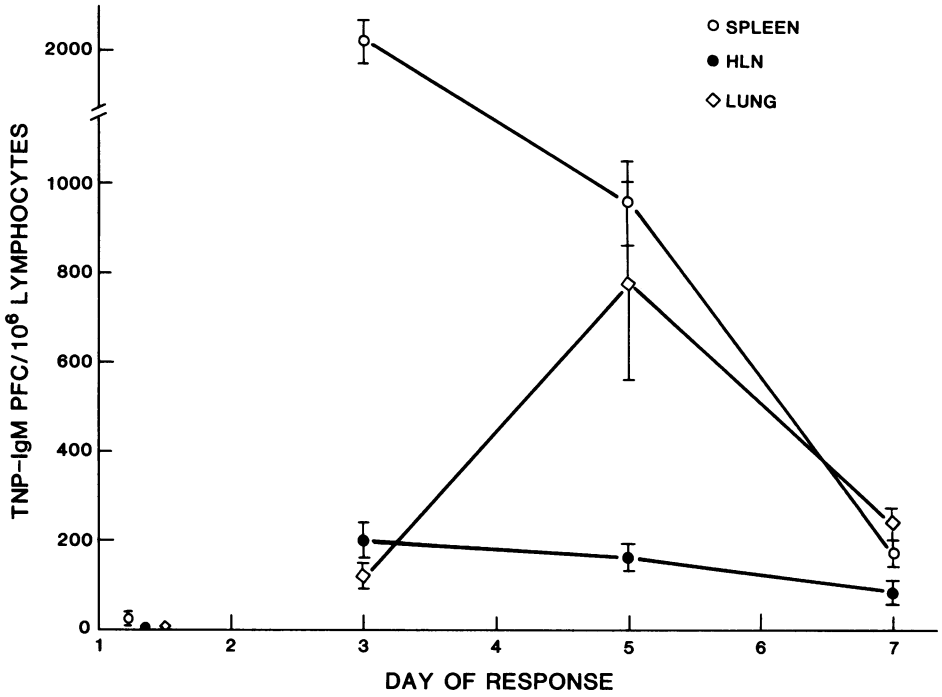


FIG. 1. Appearance of antibody-forming cells in pulmonary and systemic lymphoid tissues after a single primary i.t. immunization of mice with TNP-LPS. The dose of antigen was 20 μg per mouse. Values represent the means ± standard errors of PFC from three to six mice in each group. The responses of the saline-control mice are shown in the bottom left-hand corner.

Lab Chow) and water. The mice were periodically tested for common bacterial and viral pathogens and were found to be negative.

**Antigens.** TNP-BA was made by conjugating heat-killed *B. abortus* 1119-3 (National Veterinary Services Laboratory, Ames, Iowa) with trinitrobenzene sulfonic acid by the method of Mond et al. (24). TNP-Ficoll was prepared as described earlier (16). TNP-LPS was prepared by the method of Jacobs and Morrison (17), using LPS derived from *Escherichia coli* O111.B4 (Difco Laboratories, Detroit, Mich.).

**Immunization.** For i.t. immunization, each mouse was anesthetized with 50 to 100 μl of a 30% solution of sodium pentobarbital (Butler Co., Columbus, Ohio). A small incision was made in the neck region, the trachea was exposed, and antigen was inoculated near the distal end of the trachea with a Hamilton syringe and 30-gauge needle. The incision was stapled with surgical autoclips (Becton Dickinson and Co., Parsippany, N.J.).

**Isolation of lymphocytes.** At various times after immunization, the animals were sacrificed and different lymphoid organs were removed. Splenic lymphocytes were dispersed by pressing the spleens against the bottom of a petri dish with a disposable syringe plunger. The cell suspension was transferred to a centrifuge tube and allowed to settle so the debris could be collected at the bottom. The supernatant containing the cells was aspirated, and the cells were washed twice before use.

The HLN were collected in a petri dish with Hanks balanced salt solution, the adhering fat was gently separated, and the lymph nodes were minced finely with scissors. The resulting suspension was filtered through a nylon screen to remove debris and washed three times before use.

To collect lymphocytes from the lungs, the thoracic cavity was opened and the lungs were perfused by injecting physiological saline (containing 2 U of heparin per ml) into the right ventricle of the heart until the color of the lungs turned pale, indicating depletion of the majority of the RBC. Lung lobes were removed free of trachea and lymph nodes and cut into small pieces in Hanks balanced salt solution with scissors. The tissue fragments were then homogenized in a stomacher (Tekmar Co., Cincinnati, Ohio) for 2 min and filtered through a nylon screen. The cell suspension was washed three times and resuspended in Hanks balanced salt solution. Slides were made by cytocentrifugation and stained with Wright-Geimsa solution for differential counts.

**Hemolytic PFC assay.** Lymphocytes from different lymphoid organs were assayed for the number of direct immunoglobulin M (IgM) PFC by a glass slide modification of the method of Jerne and Nordin (19). SRBC were coupled with TNP by the procedure of Rittenberg and Pratt (36). For estimating polyclonal antibody responses, SRBC were coated with protein A (Pharmacia Fine Chemicals, Uppsala, Sweden), using chromium chloride (13). For IgM responses an appropriate dilution of rabbit anti-IgM was added to the plaquing mixture. TNP-specific IgG or IgA PFC were estimated by adding rabbit anti-mouse IgG or IgA antiserum (Nordic Immunological Laboratories, Tilburg, The Netherlands) to the plaquing mixture after inhibiting IgM PFC with goat anti-mouse IgM antiserum (Sigma Chemical Co., St. Louis, Mo.).

**Statistical analysis.** Statistical significance of the data was evaluated by the Student *t* test. The differences were considered significant if *P* values were less than 0.05.

## RESULTS

**Anti-TNP antibody responses in different lymphoid organs after immunization with TNP-LPS.** LPS is a potent mitogen and a polyclonal activator of murine splenic B cells (7).

TABLE 1. Polyclonal antibody responses in lungs and other lymphoid organs after i.t. immunization with TNP-LPS[a]

| Day after immunization | Group | Polyclonal IgM PFC/$10^6$ lymphocytes[b] | | |
|---|---|---|---|---|
| | | Spleen | HLN | Lung |
| 3 | TNP-LPS | 5,667 ± 883* | 1,117 ± 217* | 2,302 ± 714* |
| | Control | 1,670 ± 95 | 70 ± 10 | 24 ± 13 |
| 5 | TNP-LPS | 4,332 ± 469* | 1,130 ± 231* | 3,120 ± 451* |
| | Control | 2,250 ± 122 | 5 ± 1 | 10 ± 6 |
| 7 | TNP-LPS | 3,482 ± 175 | 274 ± 110* | 864 ± 579* |
| | Control | 3,107 ± 219 | 10 ± 2 | 8 ± 4 |

[a] Twenty micrograms of TNP-LPS in 40 μl of saline was injected i.t. For control mice, a similar volume of 0.85% saline was injected.

[b] PFC were assayed by using protein A-coated SRBC and adding an appropriate dilution of rabbit anti-mouse IgM antibody to the plaquing mixture. The values are means ± standard errors for two to four mice. * Significantly different ($P < 0.05$) compared with control.

When mice were immunized i.t. with TNP-LPS, there was good anti-TNP antibody responses not only from HLN and lung B cells but also from splenic B cells (Fig. 1). The antibody responses in all these lymphoid tissues were significantly higher ($P < 0.05$) than in control mice inoculated with saline. The peak response in lungs occurred on day 5; the results were significantly different ($P < 0.05$) from day 3 or day 7 data.

**Polyclonal antibody responses in pulmonary lymphoid tissues induced by TNP-LPS.** Infection with microorganisms frequently results in the polyclonal activation of B lymphocytes. For example, *Mycoplasma pneumoniae* has been found to induce polyclonal B cell activation in in vitro cultures of mouse splenic B cells and human peripheral blood cells (2, 3). Therefore, in the present study we wanted to measure such polyclonal responses in pulmonary lymphoid tissues after an i.t. immunization of mice with TNP-LPS. There are two ways by which we can measure polyclonal PFC. One procedure is by using SRBC coated with unrelated haptens like 4-hydroxy-3-indo-5-nitrophenyl acetate, fluorescein isothiocyanate, phosphoryl choline, etc. The other method is by using protein A-coated SRBC and adding rabbit anti-mouse IgM or IgG to the plaquing mixture (13). We used the latter method because it is less time consuming and more sensitive than the former.

The results shown in Table 1 indicate a significant ($P < 0.05$) increase in the polyclonal IgM antibody responses from the HLN and lung B cells on 3, 5, and 7 days after i.t. immunization. In the spleen, the polyclonal PFC remained significantly higher ($P < 0.05$) up to 5 days postimmunization. It should be pointed out that in the spleens of control mice, there was a high level of cells that secreted IgM spontaneously. This has been observed before, and such cells were referred to as natural antibody-secreting cells (32).

**TNP-BA-induced anti-TNP immune responses.** In our previous study (12) it was found that TNP-BA elicited a good antibody response from peripheral LN B cells. Therefore, TNP-BA was selected for its ability to induce an anti-TNP PFC response in pulmonary lymphoid tissues after i.t. immunization. The results indicate a significantly ($P < 0.05$) higher antibody formation in both pulmonary and systemic lymphoid tissues than in the controls. The peak antibody response in the lungs occurred on day 9 ($P < 0.05$, compared with other time points; Fig. 2).

**Antibody responses to TNP-Ficoll in pulmonary lymphoid tissues.** In contrast to the TI-1 antigens, which elicited good antibody responses in the pulmonary lymphoid tissues, the TI-2 antigen TNP-Ficoll failed to induce anti-TNP responses

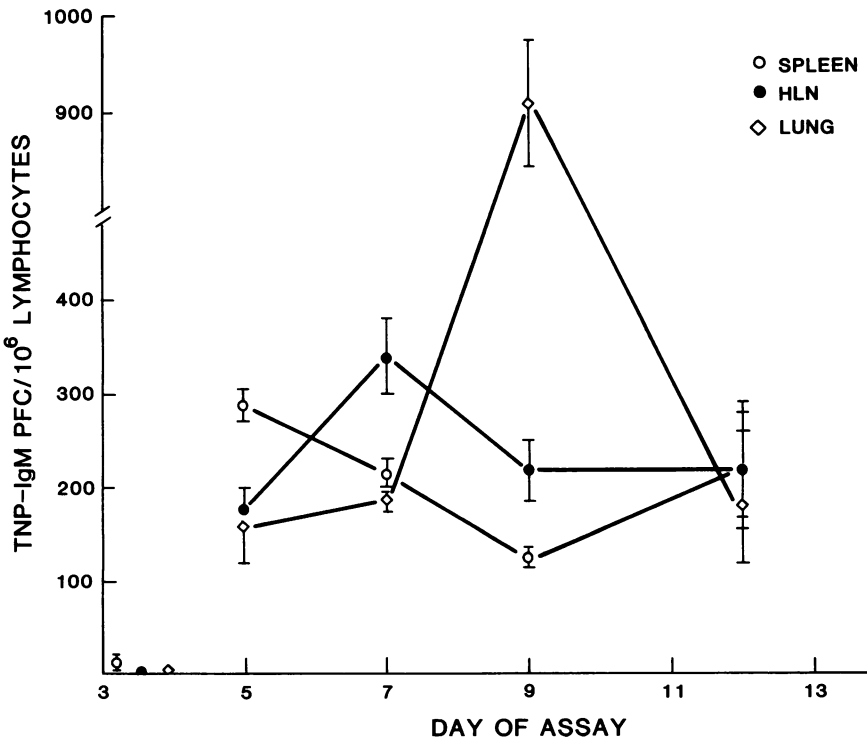


FIG. 2. Antibody responses in pulmonary and systemic lymphoid tissues after a single primary i.t. immunization with TNP-BA. The dose of antigen per mouse was $5 \times 10^7$ *B. abortus* organisms conjugated with TNP. Values represent the means ± standard errors of PFC from three to six mice in each group. The responses of the saline-control mice are shown in the bottom left-hand corner.

TABLE 2. Anti-TNP antibody responses in pulmonary lymphoid tissues after i.t. immunization of mice with TNP-Ficoll plus BA

| Day after immunization | Group | TNP-IgM PFC/$10^6$ lymphocytes[a] | | |
|---|---|---|---|---|
| | | Spleen | HLN | Lung |
| 5 | TNP-Ficoll[b] | 1,021 ± 300* | 6 ± 4 | 1 ± 1 |
| 7 | TNP-Ficoll | 779 ± 43* | 4 ± 1 | 6 ± 2 |
| 9 | TNP-Ficoll | 639 ± 61* | 3 ± 2 | 5 ± 2 |
| 12 | TNP-Ficoll | 307 ± 71* | 1 ± 1 | 4 ± 3 |
| 7 | TNP-Ficoll + *B. abortus*[c] | 1,005 ± 150* | 110 ± 21* | 114 ± 23* |
| 9 | TNP-Ficoll + *B. abortus* | 830 ± 49* | 74 ± 9* | 271 ± 34* |
| 12 | TNP-Ficoll + *B. abortus* | 570 ± 81* | 51 ± 12* | 95 ± 29* |
| | Saline control | 10 ± 6 | 2 ± 1 | 1 ± 1 |

[a] PFC were assayed by using TNP-coated SRBC. Values are means ± standard errors for six to eight mice in each group.

[b] Twenty micrograms of TNP-Ficoll was injected i.t. in a volume of 40 μl of saline per mouse.

[c] Mice were injected i.t. with TNP-Ficoll (20 μg per animal) mixed with about $5 \times 10^7$ unconjugated *B. abortus* cells in a total volume of 40 μl per mouse. *, Significantly different ($P < 0.05$) compared with saline control.

from the HLN and lung B cells of mice even up to 12 days after an i.t. immunization. However, these animals exhibited an excellent PFC response from their splenic B cells (Table 2). There were no IgG or IgA PFC responses in the pulmonary lymphoid tissues of these mice (data not shown).

**Immunization of mice with TNP-Ficoll plus *B. abortus* induces antibody responses in pulmonary lymphoid tissues.** Since TNP-Ficoll alone was unable to induce antibody responses in pulmonary lymphoid tissues, we immunized mice with TNP-Ficoll mixed with unconjugated *B. abortus*, by the i.t. route. This resulted in a statistically significant ($P < 0.05$) increase in anti-TNP PFC from both HLN and lung B cells over that for the saline controls. The peak response in lungs was on day 9, and it was significantly different ($P < 0.05$) from the day 7 or day 12 response (Table 2).

**Inflammation in lungs induced by different TI antigens.** To test whether the ability of an antigen to induce inflammation in the respiratory tract is related to the appearance of antibody responses in the lung, we examined lung homogenates for the presence of neutrophils, eosinophils, (polymorphs), monocytes, etc. The results show an enhancement ($P < 0.05$) in the number of polymorphs, monocytes, and lymphocytes in TNP-LPS- and TNP-BA-immunized mice

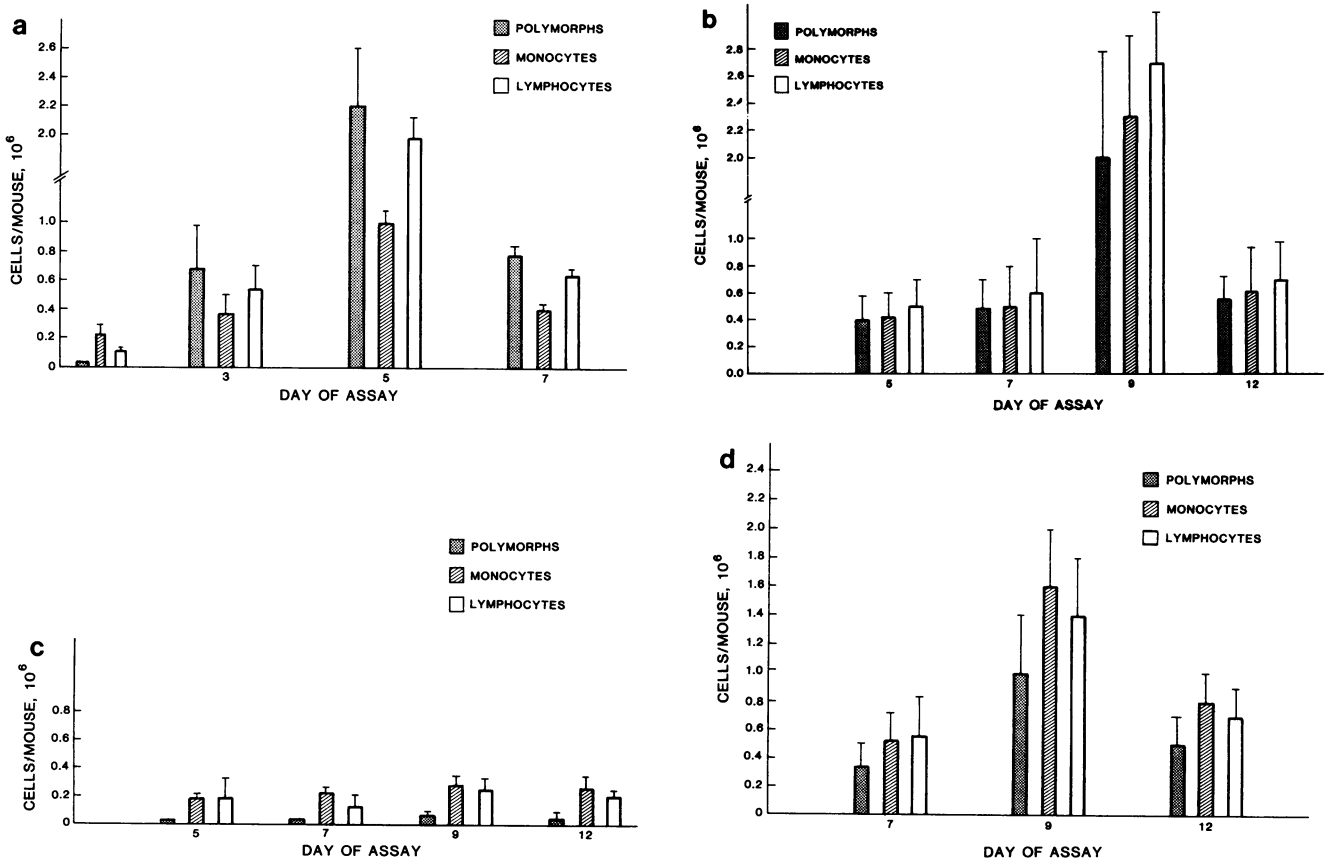


FIG. 3. Total number of different types of lymphoid and myeloid cells present in the lung homogenates of mice i.t. immunized with different antigens. (a) Animals were immunized with 20 μg of TNP-LPS per mouse. (b) Animals were immunized with TNP-BA. The dose of antigen per mouse was $5 \times 10^7$ *B. abortus* organisms conjugated with TNP. (c) Animals received 20 μg of TNP-Ficoll per mouse. (d) Animals received 20 μg of TNP-Ficoll mixed with $5 \times 10^7$ unconjugated *B. abortus* organisms per mouse. On the day of the assay, animals were sacrificed, the lungs were processed as described in Materials and Methods, and the lung cell suspension was stained with Wright-Giemsa stain for differential counts. The values represent the means ± standard errors of the different cell populations per mouse. The cell counts from the lungs of saline-control animals are shown in the lower left-hand corner of panel a. Three to six mice were used for each time point in the TNP-LPS- and TNP-BA-immunized groups, and 6 to 10 mice were used for each time point in the TNP-Ficoll and TNP-Ficoll-plus-BA groups.

compared with saline controls (Fig. 3a and b). The biggest increase in lymphocytes coincided with the peak of the PFC response. But the TI-2 antigen TNP-Ficoll, which failed to induce anti-TNP PFC in the lymphoid tissues of the respiratory tract, did not cause an increase ($P > 0.05$) in the number of granulocytes or monocytes (Fig. 3c). However i.t. immunization of mice with TNP-Ficoll plus *B. abortus* resulted in a significant ($P < 0.05$) increase in the number of inflammatory cells and anti-TNP PFC in the lung (Fig. 3d).

## DISCUSSION

The most significant finding of this investigation was the appearance of antibody-forming cells within the lung and HLN B cells after a primary i.t. immunization of mice with the TI-1 antigens TNP-LPS and TNP-BA but not with the TI-2 antigen TNP-Ficoll. Both polyclonal and anti-TNP PFC were found as early as 3 days after the administration of TNP-LPS. This appears to be a unique property of TI-1 antigens, since in previous studies which employed thymus-dependent antigens, the primary responses in the lungs and HLN were small and appeared late (21–23).

What is the basis for the appearance of effector B cells in lungs after a single immunization with TI antigens? This is a difficult question to resolve at this time, and we cannot as yet exclude one possibility over another. One reason for such a rapid appearance of antibody responses in the TNP-LPS-immunized mice might have been the stimulation of resident B cells in the HLN and lung parenchyma by the LPS. Alternatively, LPS could have caused the migration of antigen-specific B cells or plasma cells into the lung as a result of inflammation. Our cytological examination of lung homogenates showed the presence of inflammation in the lung parenchyma of animals immunized with TNP-LPS (Fig. 3). Other investigators have also demonstrated the occurrence of inflammation in the respiratory tract of animals inoculated with LPS (9, 14).

i.t. immunization with TNP-BA resulted in similar antibody responses in both pulmonary and systemic lymphoid tissues, although the peaks of the antibody responses in the lungs were different than those in TNP-LPS-immunized mice. This might have been due to the structural and functional differences between the *E. coli*-derived LPS and the *B. abortus*-derived LPS (25, 26).

An interesting result of this study was the failure of TNP-Ficoll to induce a PFC response from the HLN and lung B cells despite a good response from the splenic B cells of the mice. Since TNP-Ficoll is a soluble antigen, its unresponsiveness in pulmonary lymphoid tissues may be due to the poor retention of the antigen in the pulmonary tissues or to the failure of TNP-Ficoll to induce inflammation in the lungs. Our present results confirm the absence of inflammation in the lungs of TNP-Ficoll-immunized mice. The antibody response to TNP-Ficoll in pulmonary tissues was restored when the mice were immunized with TNP-Ficoll mixed with *B. abortus*. This shows the importance of the inflammatory agents or adjuvants in the antibody responses to soluble antigens. This observation is in agreement with our earlier study, in which popliteal and inguinal LN B cells responded to TNP-Ficoll when mice were injected in the footpads with TNP-Ficoll plus *B. abortus* (12). A previous study by others has shown that the concentration of soluble protein antigens retained in the HLN was 10 times more in animals treated with inflammatory agents such as *Mycobacterium bovis* BCG or carrageenan than in normal controls (33). Also, inhalation of antigens into inflammed but not normal lungs resulted in the development of high levels of circulating antibodies and activated T cells in the lungs of rabbits (42).

The absence of inflammation in the lungs may not be the only reason for the lack of TI-2 antigen-specific PFC responses in pulmonary lymphoid tissues, because another soluble TI-2 antigen, polyvinylpyrrolidone (PVP), failed to elicit anti-PVP PFC responses from the lung and HLN B cells even after mice were immunized i.t. with PVP mixed with unconjugated *B. abortus* (data not shown). Also, there was no antibody response from popliteal and inguinal LN B cells of mice immunized subcutaneously in the footpads with PVP plus *B. abortus* (Goud et al., in press). This observation is in contrast to the data for TNP-Ficoll plus *B. abortus*. Currently we are attempting to analyze the role of various cells in the antibody responses of TI-2 antigens in pulmonary lymphoid tissues by performing in vitro cell mixing experiments with accessory, B and T cells derived from the spleen, HLN, and lung.

Studies of the pulmonary immune responses to TI antigens has relevance to the development of vaccines for human respiratory pathogens like pneumococci, *Hemophilus influenzae*, etc. TNP-Ficoll is a prototype TI-2 antigen, a category to which a variety of bacterial polysaccharides belong. Our present study shows that TNP-Ficoll induces an antibody response in the pulmonary lymphoid tissues only when it is administered with *B. abortus*. But such use of particulate adjuvants may not be feasible in human clinical trials. One alternative possibility is combined immunization by the oral and intranasal routes. This procedure was found to be effective in conferring protective immunity to both the upper and lower respiratory tract of mice against Sendai virus and pertussis toxin (31, 37). The rationale behind this method is that the oral immunization primes the B lymphocytes from the gut-associated lymphoid tissues such as mesenteric lymph nodes and Peyer's patches and these activated cells migrate to the respiratory tract upon secondary challenge (40). In this regard we have found that, in contrast to peripheral LN (axillary, brachial, popliteal, and inguinal) cells, the B lymphocytes from mesenteric lymph nodes make good anti-TNP PFC responses to TNP-Ficoll in in vitro culture experiments (Goud et al., in press). Therefore, it will be interesting to study the antibody responses in the lung after oral and i.t. immunization with TNP-Ficoll; such studies are in progress.

In conclusion, the results of the present study indicate that after a primary i.t. immunization with the TI-1 antigens TNP-LPS and TNP-BA, there were antigen-specific and polyclonal antibody responses in pulmonary lymphoid tissues. In contrast, the TI-2 antigen TNP-Ficoll failed to elicit such a response. The antibody response to TNP-Ficoll was restored in the lung and HLN B cells when mice were immunized with TNP-Ficoll mixed with *B. abortus*.

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