# Development of Cellular and Humoral Immunity in the Respiratory Tract of Rabbits to *Pseudomonas* Lipopolysaccharide

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ABSTRACT Immunization with *Pseudomonas* lipopolysaccharide induced both cellular and humoral immunity in rabbits, particularly in the respiratory tract after intranasal immunization. Either parenteral (i.m.) or intranasal immunization elicited an IgG antibody response in respiratory secretions, but only intranasal immunization produced secretory IgA antibody. Immunization by both routes stimulated serum IgM and IgG agglutinative antibodies. Because both methods of immunization produced skin test reactivity which had components of both Arthus and tuberculin-like reactions, cellular immunity was more readily assessed by the measurement of migration inhibitory factor (MIF) released from immune lymphocytes in respiratory and spleen cell suspensions after challenge with the lipopolysaccharide antigen. After intranasal vaccination, MIF activity was detected in the respiratory tract by direct assay; in contrast, i.m. immunized rabbits did not produce respiratory MIF. Both modes of immunization resulted in splenic MIF activity. However, lymphocytes were only capable of producing MIF for short periods after primary immunization had ended, apparently losing this function in about 2–3 wk. Therefore, it was concluded that cellular immunity by in vitro assay was transient after primary immunization with this *Pseudomonas* antigen in contrast to the more persistent humoral immunity. The biological significance of immune lymphocytes as part of the coordinated host defense of the lung needs further evaluation.

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## INTRODUCTION

Previous animal studies have shown that cellular immunity or delayed hypersensitivity occurs in the respiratory tract after appropriate antigen contact. Intranasal immunization of guinea pigs with dinitrophenylated human IgG (DNP-HGG) [1] induced immune respiratory lymphocytes which could produce migration inhibition factor (MIF) when stimulated with DNP-HGG (1, 2). Studies of pulmonary infection with *Mycoplasma pneumoniae* in hamsters (3, 4) or with such facultative intracellular organisms as Calmette-Guérin bacillus in rabbits (5) and *Listeria monocytogenes* in mice (6) have demonstrated that delayed hypersensitivity was a major component of acquired immunity. Recently, extracts from group A streptococci were shown to stimulate lymphocytes to inhibit migration of guinea pig alveolar macrophages (7).

Certain gram-negative bacilli cause infections in humans which elicit prominent mononuclear cell responses. It has been suggested that the cellular response to these gram-negative bacterial infections might be an expression of delayed hypersensitivity or cellular immunity. In infections caused by *Salmonella typhosa* mononuclear cells infiltrate lesions of the skin, lungs, and other viscera (8) and appear in the diarrheal stools (9). Histological studies of human lungs after fatal gram-negative pneumonias have identified appreciable infiltrations of mononuclear cells. The presence of mono-

[1] *Abbreviations used in this paper:* DNP-HGG, dinitrophenylated human IgG; FCS, fetal calf serum; LPS-II, lipopolysaccharide immunotype 2; MHS, modified Hank's salt solution; MIF, migration inhibitory factor; PMN, polymorphonuclear.

nuclear cells was particularly noticeable in cases of pneumonia caused by *Escherichia coli* (10, 11), *Klebsiella pneumoniae* (12), and *Pseudomonas aeruginosa* (13). Recently, normal humans were shown to have delayed or tuberculin-like skin reactivity to a variety of bacterial endotoxins which suggested that they had natural or pre-existing cellular immunity (14).

Previously we examined the interaction of respiratory opsonic antibodies and alveolar macrophages in rabbits immunized with whole-cell *P. aeruginosa* vaccines (15, 16). The present work extends these observations to the development of cellular immunity in respiratory tract after immunization with lipopolysaccharide extracted from a strain of *P. aeruginosa*.

## METHODS

*Pseudomonas antigen.* Lipopolysaccharide was acid extracted and purified (17) from a strain of *P. aeruginosa*, immunotype 2 in the Fisher typing scheme (18). The chemical properties of this lipopolysaccharide antigen (LPS-II) have been analyzed (17); it contained 5.4% bacterial protein. The antigen (Parke, Davis & Company, Detroit, Mich., P.D. lot ×41462) was supplied as an aqueous mixture at 4.8 mg/ml concentration and was stored at 4°C. A lyophilized culture of the original *P. aeruginosa* strain (P.D. no. 05142) was obtained and the biochemical properties and antibiotic sensitivities of the microorganism was studied.[2] The organism's growth was inhibited by 3 μg/ml concentration of gentamicin.

*Animals.* Out-bred New Zealand white rabbits (2–3 kg) were used. Before immunization with LPS-II antigen, all animals had negative skin reactivity to a 0.48 μg/0.1 ml intradermal tests, a hemagglutinative antibody titer to LPS-II of 1:4 or less, and negative nasal cultures for *Bordetella bronchiseptica*. Only 25% of the rabbits screened with nasal cultures were not colonized with this microorganism.

*Immunization schedules.* Rabbits were immunized with LPS-II either intramuscularly (i.m.) or intranasally (15). i.m. vaccinated rabbits received LPS-II at a dose of 20 μg/kg given in a lateral thigh three times a week on alternate days for 2 wk (total six injections). For intranasal immunization, rabbits received 240 μg of LPS-II in a 2-ml volume of saline three times a week for 2 wk.

Blood samples were taken at various intervals during and after immunization; animals were finally sacrificed and bronchial washings, spleens, and skin samples were obtained. The vaccination course for some rabbits was limited to three doses of antigen and they were sacrificed on the 7th day. Booster injections of LPS-II were not given.

*Preparation of bronchial secretions and source of respiratory and spleen cells.* General methods for pulmonary lavage and respiratory cell cultures were the same as used previously (15, 16). About 150 ml of modified Hank's salt solution (MHS)[3] was used to lavage each pair of lungs. The lavage fluid was centrifuged to pellet respiratory cells and the supernatant fluid was decanted. The supernatant fluid was rapidly concentrated with positive pressure ultrafiltration (Amicon Corp., Lexington, Mass.) using an UM-10 filter, dialyzed against borate-saline buffer, and finally concentrated with negative pressure dialysis to a 1-ml volume. This fluid was designated as concentrated bronchial secretions.

The cell pellet was resuspended in MHS without heparin or serum and washed and centrifuged twice before the cells were finally suspended in 2 ml of modified McCoy's 5A media (Grand Island Biological Co., Grand Island, N. Y.) enriched with 1% glutamine and 10% heat-inactivated fetal calf serum (FCS). Cell viability was determined by dye exclusion. Because respiratory cells usually contained less than 5% erythrocytes in the pellet, they were not subjected to hypotonic lysis. Phagocytic cells in the respiratory cell suspensions were differentiated from lymphocytes by their morphology in Wright's stained smears, ingestion of polystyrene latex balls (mean diameter 1.1 μm), and staining with neutral red dye (19).

The spleens were minced, homogenized in a glass grinder containing MHS, and washed through several layers of loose gauze to remove splenic debris. The cell suspension was centrifuged at 1,800 rpm for 5 min at 25°C. Red cells in the pellet were hypotonically lysed[4] within a 5-min interval; the spleen cells were then washed several times in MHS and suspended in 2–3 ml of enriched McCoy's media.

*Fractionation of cell suspensions.* Respiratory and spleen cell suspensions were filtered through nylon-wool-glass bead columns as previously described (20). The columns were equilibrated with enriched McCoy's media and cell suspensions, applied to individual columns, were allowed to incubate for 30 min. Nonadherent cells were eluted at a flow rate of about 0.5 ml/min during a 30-min interval. The eluted cells were centrifuged and washed once before a final suspension was made.

*Preparation of MIF containing culture fluids.* Column purified splenic lymphocytes were cultured in enriched McCoy's media to which 5 μg/ml of gentamicin sulfate was added. The cell density was $5–6 \times 10^6$ cells/ml and the volume was 2.2 ml. Sets of tubes contained either no antigen or LPS-II at final concentrations of 10 μg/ml or 24 μg/ml. After 24 h of culture at 37°C in 5% $CO_2$ and moist air, cell-free supernates were collected and used in alveolar macrophage migration assays.

Respiratory cell suspensions were incubated in similar media at a density of $1–2 \times 10^6$ lymphocytes/ml. Cultures were established with no antigen and with LPS-II added to a final concentration of 24 μg/ml. Cell-free supernates were collected at 24 h and added to alveolar macrophage-containing chambers for assay of MIF activity.

*MIF assay.* Inhibition of macrophage migration was evaluated by the method of David, Al-Askari, Lawrence, and Thomas (21). Each Sykes-Moore chamber, containing two capillary tubes packed with a macrophage cell suspension, was filled with approximately 0.9 ml of test media and incubated at 37°C in moist air and 5% $CO_2$. Duplicate chambers were used for each assayed specimen. The areas of macrophage migration were drawn at 24 h and the mean area of migration ±standard error for four capillary tubes was calculated. The percent of migration inhibition

[2] Kindly performed by Dr. E. Ryschenkow, Microbiology Section, Department of Clinical Pathology, NIH, Bethesda, Md.

[3] Prepared by NIH Media Unit without calcium or magnesium ions and phenol red.

[4] Prepared by NIH Media Unit. The stock solution contained ammonium chloride 33.1 g, potassium bicarbonate 4 g, and EDTA 14.88 g in 4 liters of water.

TABLE I

*Antibody Response in Bronchial Secretions and Serum after Intranasal or Intramuscular Immunization with LPS-II*

| | Nonimmunized controls | 1 wk* | 2 wk‡ | 3 wk | 4 wk |
|---|---|---|---|---|---|
| Intranasal | | | | | |
| Number rabbits | 3 | 5 | 4 | 4 | 3 |
| Bronchial secretions | <2§ (0–2) | <2 (0–4) | 2.8 (0–16) | 8.0 (4–16) | 12.7 (8–32) |
| Whole serum | 2.5 (2–4) | 111.4 (4–1024) | 215.3 (64–1024) | 1712.0 (1024–4096) | 1290.2 (1024–2048) |
| Reduced serum‖ | <2 (0–2) | 4.0 (2–8) | 13.5 (4–256) | 128.0 (32–256) | 256.0 (128–512) |
| Intramuscular | | | | | |
| Number rabbits | 3 | 3 | 5 | 5 | 3 |
| Bronchial secretions | <2 (0–2) | <2 (0–2) | 12.1 (4–32) | 9.2 (4–32) | 8.0 (4–16) |
| Whole serum | 5.0 (4–8) | 322.5 (256–572) | 3104.2 (512–8192) | 2048.0 (1024–4096) | 645.1 (512–1024) |
| Reduced serum | <2 (0–2) | 3.2 (2–4) | 111.4 (16–512) | 194.0 (64–512) | 64.0 (32–128) |

* After three doses LPS-II.

‡ After six doses LPS-II.

§ Geometrical mean of hemagglutinative antibody titers for the group and titer range observed.

‖ Reduced with 2-mercaptoethanol.

was derived from the formula:

$$\text{migration inhibition} = 1 - \frac{\text{mean area of migration with LPS-II}}{\text{mean area of migration without LPS-II}} \times 100.$$

Each macrophage assay included two sets of control chambers containing: (*a*) freshly prepared enriched media and (*b*) supernatant material from nonstimulated lymphocyte cultures to which a concentration of 24 μg/ml LPS-II was added at the time of assay. The particular macrophage assay was considered valid and the data included in Results only if macrophage migration in the chambers containing supernatant media from nonantigen-incubated cell cultures were equal to the above mentioned controls. Significant inhibition of macrophage migration was considered evident when migration inhibition was 20% or greater (22).

The direct MIF assay utilized the respiratory cell suspensions obtained from control or immunized rabbits. These cells contained approximately 92% macrophages and were consistently greater than 95% viable. The average cell yield was about $4–6 \times 10^7$ cells per pair of lungs. Cells were washed twice and resuspended to a final concentration of $2 \times 10^7$ macrophages/ml in enriched McCoy's media which contained gentamicin at 5 μg/ml before 50-μl capillary tubes were filled. The remainder of the cell suspensions were fractionated on wool-glass bead columns to obtain lymphocytes for cell cultures as described.

For the indirect MIF assay, cell-free supernatant material was obtained from antigen-stimulated and nonstimulated cultures of column separated lymphocytes of spleen and respiratory tract origin. Alveolar macrophages used in these indirect assays were lavaged from nonimmunized normal rabbit lungs. The cell composition was the same as that obtained from immunized animals; macrophages were adjusted to $2 \times 10^7$ cells/ml before capillary tubes were filled.

*Antibody measurements.* Hemagglutination antibodies to LPS-II were measured in concentrated bronchial secretions and sera from control and immunized rabbits with a described method (23). The titer was the reciprocal of the highest dilution showing one plus red cell agglutination. Duplicate hemagglutination titers using nonsensitized red cells were run for each specimen; an antibody preparation of known titer was included with each assay as a positive control. 2-Mercaptoethanol reduction of serum macroglobulins was done as outlined (24).

*General immunological methods.* Antisera to various rabbit proteins and immunoglobulins have been identified previously (15). Serum and bronchial secretions were separated in 5 ml 10–40% linear sucrose density gradients prepared in borate-saline buffer, pH 8.0, and centrifuged at 35,000 rpm in a SW-39 rotor for 18 h at 4°C (25). Marker proteins included in each gradient were a 7S rabbit IgG isolated from colostrum and labeled with $^{125}I$ (26) and 11S crystalline beef liver catalase (Worthington Biochemical Corp., Freehold, N. J.). Catalase activity was determined by the method of Allen, Sirisinha, and Vaughan (27) and expressed as the change in absorbancy per minute at 230 nm.

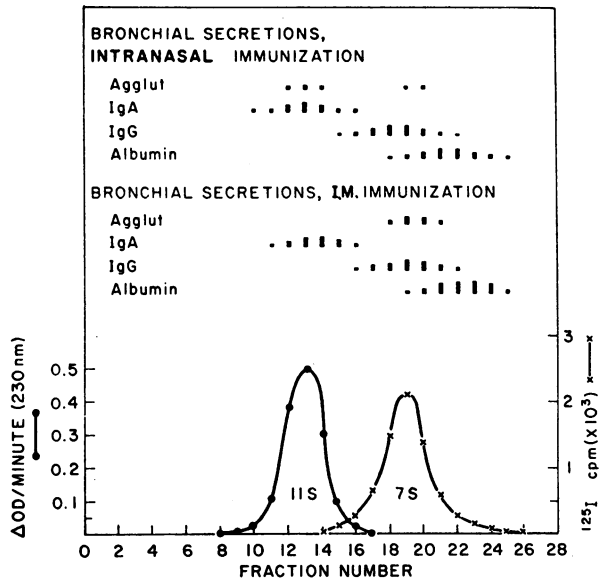


FIGURE 1 Bronchial secretions obtained from an intranasally and an i.m. immunized rabbit 1 wk after completed immunization with LPS-II were simultaneously separated in sucrose density gradients by ultracentrifugation. Fractions from each gradient were comparable and the positions of the 11S and 7S markers were the same in each gradient. The position of the 11S catalase marker (●) was determined enzymatically and is plotted as ΔOD/min; the radiodinated 7S marker position (×) is shown as counts per minute (cpm). The relative concentrations of albumin, immunoglobulins, and hemagglutinative antibody activity in each gradient fraction are denoted by vertical bars.

*Skin testing and histology.* Before immunization, rabbits were screened for pre-existing skin reactivity to the LPS-II antigen with a 0.48 μg/0.1 ml intradermal test. This intradermal dose was insufficient to cause a rise in the hemagglutination antibody titer. All skin tests were placed in shaved skin areas over the flanks and were observed at 4, 24, and 48 h. A positive test had erythema and induration of $> 0.6$ cm² and increased skin thickness of 0.2 mm or greater. Approximately 48 h before sacrifice of an animal, a battery of skin tests was applied which included a saline diluent and LPS-II at 0.048, 0.48, and 4.8 μg/0.1 ml. 4 h before sacrifice, the battery was repeated on the opposite flank. At necropsy, skin samples were fixed in picric acid and formaldehyde (Bouin's solution); hematoxylin and eosin slides were made. Slides were graded for the degree of cellular infiltration in the dermal layer and around vascular structures; a percentage estimate for the proportion of polymorphonuclear and round cell or mononuclear cell infiltration was given to each section.

## RESULTS

The following data were collected from 47 rabbits immunized with LPS-II either i.m. or intranasally and 20 control rabbits. The immunized and control rabbits were comparable in size (mean±SEM, 2.42 kg±0.09). Their extirpated lungs (12.8 g±0.3) and spleens (1.56 g±0.11) were comparable as well. Immunization with LPS-II apparently had no adverse effects; weight gain was progressive and local reactions at parenteral injection sites were not detected. Intranasally and i.m. immunized rabbits had equivalent number of recoverable respiratory and spleen cells and identical cell differential counts.

*Antibody responses.* In Table I, hemagglutinative antibody titers to LPS-II are shown after i.m. and intranasal immunization. For serum specimens, total and 2-mercaptoethanol-resistant agglutinative titers are given to differentiate the proportions of macroglobulin (IgM) and IgG antibody present. Separation of IgM and IgG fractions in sera by gel filtration methods previously used to identify agglutinative antibody activity in immune rabbit sera (15) confirmed the reliability and specificity of these titers. For concentrated bronchial secretions, only total agglutinative titers were done because of the absence of IgM immunoglobulin in normal respiratory secretions (15).

As shown in Table I, serum antibody titers were detected at 1 wk, or after three doses of LPS-II, in both groups of immunized rabbits, but antibody was principally confined to the IgM class. At 2 wk when the immunization course was completed, i.m. immunized rabbits had higher serum agglutinative titers in both IgM and IgG fractions than did the intranasally immunized animals. However, by the 3rd wk serum titers were comparable in both groups. Agglutination in bronchial secretions was not detected before 2 wk and generally was proportional to the serum IgG titer, but the immunoglobulin classes in the bronchial secretions having antibody activity for LPS-II were different depending upon the route of immunization. Five paired bronchial secretion specimens from i.m. and intranasally immunized rabbits obtained 1 wk after immunization were examined in sucrose density gradients. In Fig. 1, a representative comparison of agglutinative activity and the quantitation of protein components in the various gradient fractions are shown in the bronchial secretions. The two gradients had comparable fraction numbers and the position of the 11S and 7S markers were identical. Agglutinative activity in the bronchial secretions from the intranasally immunized rabbit was present in the IgA and IgG fractions; whereas, in the bronchial specimen from the i.m. immunized rabbit, agglutination was confined to the IgG fractions. Similar gradients showed agglutinative activity in serum specimens to be present in both IgM and IgG fractions from i.m. and intranasally immunized rabbits.

*Skin tests.* Coincident with the development of agglutinative antibodies, positive skin reactivity to intradermal doses of LPS-II was elicited in all rabbits in both the i.m. and intranasal groups that had received at least three doses of antigen (number tested, 40).

TABLE II

*Lymphocyte Yields from Column Fractionation of Respiratory and Spleen Cell Suspensions**

| No. cells in original suspension | Viability | Lymphocytes in differential count | No. lymphocytes obtained after fractionation | Lymphocyte recovery | Lymphocytes in differential count | Viability |
|---|---|---|---|---|---|---|
| | % | % | | % | % | % |
| Respiratory cells | | | | | | |
| $5.2 \times 10^7 \pm 0.3$‡ ($1.7 \times 10^7$–$1.3 \times 10^8$)§ | >95 | 7.2±0.6 (3–15) | $2 \times 10^6 \pm 0.3$ ($0.6$–$5.1 \times 10^6$) | 46±6.8 | 36±4.8 | >95 |
| Spleen cells | | | | | | |
| $1.9 \times 10^8 \pm 0.6$ ($1.4$–$3 \times 10^8$) | 78±1.7 | 71±2.6 (60–80) | $5.0 \times 10^7 \pm 0.43$ ($1.6$–$8.7 \times 10^7$) | 38±3.3 | 86±2.8 | 83±2.1 |

* From paired respiratory and spleen cell suspensions of 24 rabbits.

‡ Mean±SEM.

§ Range observed.

The pattern of skin reactivity, particularly to the 4.8 μg/0.1 ml challenge, resembled a type III or Arthus reaction (28). Skin erythema and induration developed within 4–6 h and the cellular reaction, histologically, was primarily polymorphonuclear (PMN). At 24 h mononuclear cell infiltration had increased in the inflammed skin and the reaction was observed to fade in the next 24 h period. Skin reactivity was proportional to the humoral antibody titer. However, the relative proportions of PMN and mononuclear cells were very dependent upon the dose of intradermal antigen, as previously discussed (14). When the minimal reactive dose, i.e. 0.48 or 0.048 μg, was used, the proportion of mononuclear cells increased. With the smaller antigen dose the histological pattern of the evolving skin tests in the immune animals more closely resembled a turberculin-like or delayed reaction. However, this mixed type of skin reactivity to LPS-II did not permit easy discrimination between an immediate and a delayed type of reaction. Therefore, another indicator of cellular immunity, MIF production, was measured.

*MIF.* Lymphocytes were separated from respiratory and spleen cell suspensions by column fractionation and established in cell cultures. Supernatant fluids from these cultures were the source of MIF activity in the indirect MIF assay.

Because column fractionation has not been used as a method for separating lymphocytes from respiratory cell suspensions, the efficiency of nonadherent lymphocyte recovery from respiratory cell mixtures was of interest and is contrasted with spleen cell fractionations in Table II. Data are presented from 12 i.m. and 12 intranasally immunized rabbits. Because no significant variation in the total and differential cell counts in the bronchial lavage fluid or spleen suspensions were found between the two groups, the data have been combined for presentation.

Column fractionation resulted in approximately a fivefold enrichment of respiratory lymphocytes. The remaining cells collected from the column were macrophages, but they tended to be of small size (10–15 μm diameter) yet retained their capability for surface adherence and phagocytosis. Fractionation of spleen cell suspensions separated about 38% of the original small lymphocyte population and yielded cells which had improved viability. Previously, it has been shown that nonadherent lymphocytes fractionated from guinea pig lymph node cell suspensions on similar columns were primarily T lymphocytes (20). However, differentiation of the nonadherent rabbit lymphocytes into B- and T-cell populations was not attempted in our studies.

Table III shows the degree of macrophage inhibition from direct and indirect MIF assays in rabbits immunized i.m. and intranasally with LPS-II. Evidence of MIF activity was not convincingly detected in either group of animals until 2 wk of immunization had passed with the exception of splenic lymphocyte MIF production in intranasally immunized rabbits. At 2 wk the direct MIF assays of respiratory cells from intranasally immunized rabbits were significant in five or seven animals tested and were still positive 1 wk later (four of five rabbits). The degree of inhibition obtained with indirect assays was uniformly less and probably reflected the smaller number of respiratory tract lymphocytes stimulated to obtain material for the indirect MIF assay. Splenic lymphocyte MIF was significant in rabbits tested at the 3rd week, but it was surprising that splenic MIF activity was not greater in the seven animals tested at 2 wk (four of seven did not reach 20% inhibition). Little macrophage inhibition was detected in animals at 4 wk which was 2 wk

TABLE III

*MIF Activity Produced by Respiratory and Spleen Cells after Intranasal or Intramuscular Immunization with LPS-II Using Direct and Indirect Assay**

| | Nonimmunized controls | 1 wk‡ | 2 wk§ | 3 wk | 4 wk |
|---|---|---|---|---|---|
| Intranasal | | | | | |
| Number rabbits | 4 | 4 | 7 | 5 | 3 |
| Respiratory: | | | | | |
| Direct | 0 | 8.8±2.0‖ (3–12) | 31.7±6.7 (4–54) | 26.4±9.6 (2–55) | 6.6±1.7 (5–10) |
| Indirect | 0 | 10.3±2.7 (6–15) | 15.5±4.9 (5–27) | 19.0±7.8 (5–38) | 9.0±5.6 (2–20) |
| Spleen: indirect | 6.3±1.2 (5–10) | 35.5±9.9 (15–60) | 16.8±4.7 (6–28) | 30.0±3.5 (24–40) | 5.0±0 (–) |
| Intramuscular | | | | | |
| Number rabbits | 3 | 2 | 4 | 5 | 3 |
| Respiratory: | | | | | |
| Direct | 0 | 3.3±3.0 (0–6) | 5.0±0.8 (3–7) | 16.0±6.8 (5–42) | 15.0±5.0 (10–20) |
| Indirect | 1.7±1.7 (0–5) | 7.5±2.5 (5–10) | 16.8±4.5 (10–20) | 6.8±2.1 (3–15) | 12.5±7.5 (5–20) |
| Spleen: indirect | 8.3±1.7 (5–10) | 18.0±8 (10–26) | 32.5±10.1 (15–60) | 23.0±2.6 (17–30) | 22.5±2.5 (20–25) |

* Results given for lymphocyte stimulation with 24 μg LPS-II concentration only.

‡ After three doses LPS-II.

§After six doses LPS-II.

‖ Percent alveolar macrophage inhibition expressed as the mean±SEM for each group and range of inhibition observed for each group.

after intranasal immunization was completed. In contrast, groups of i.m. immunized rabbits tested at weeks 2, 3, and 4 had significant amounts of spleen-derived MIF (10 of 12 rabbits in the three groups), yet they had less evidence of respiratory cell MIF activity by either the direct or indirect assays (only 3 of 12 rabbits produced significant inhibition). These results indicated that the intranasal method of immunization produced a greater degree of macrophage inhibition by respiratory cells as measured with the direct method; whereas, either route of immunization produced enough systemic absorption of the LPS-II antigen to significantly sensitize spleen lymphocytes.

Several i.m. immunized rabbits tested for stimulation of splenic MIF at 4 or 6 wk after immunization failed to produce significant macrophage inhibition. Therefore, the cellular immune response to primary immunization with LPS-II was short and lasted only several weeks. As mentioned before, these rabbits were not given booster doses of LPS-II antigen to restimulate MIF activity and to test cellular memory.

## DISCUSSION

Rabbits were immunized with lipopolysaccharide extracted from a strain of *P. aeruginosa,* immunotype 2, to study the development of cellular and humoral immunity, particularly in the respiratory tract after intranasal immunization. This antigen, LPS-II, was selected because it is a component of the heptavalent *Pseudomonas* vaccine (17) which is being used to immunize human subjects.

Both intranasal and parenteral methods of immunization with LPS-II produced good levels of serum antibodies and detectable amounts of respiratory antibodies. Antibody activity in bronchial secretions was usually not evident before day 14 when the entire immunization regimen had been completed. However, the immunoglobulin classes of antibody in the bronchial secretions were quite different depending upon the route of immunization. After i.m. immunization bronchial secretions contained only IgG agglutinative antibody; whereas, after intranasal vaccination, aggluti-

nation was present in the secretory IgA and IgG fractions.

Skin reactivity to intradermal testing with LPS-II was principally an Arthus reaction, reflecting the presence of circulating antibodies. However, skin test histology did not permit easy differentiation between the antigen-antibody-complement mediated cell infiltration of the Arthus reaction and the delayed response which would identify the contribution of immune cells. Therefore, MIF production by lymphocytes, obtained from spleen and respiratory sources, was examined as another correlate of developing cellular immunity and was used to corroborate the skin test results (29–31).

MIF activity was produced by respiratory cells from 75% of the intranasally immunized rabbits, as measured with the direct MIF assay, but was only detected in 25% of the respiratory tracts of parenterally immunized animals by either method of MIF assay. MIF derived from splenic lymphocytes was present by indirect assay in both vaccinated groups. The capacity of lymphocytes from fully immunized rabbits to respond to LPS-II stimulation by producing MIF activity was clearly greater than that of nonimmunized controls. Therefore, this difference was interpreted as a specific response accruing from immunization. Of considerable interest was the short interval after primary immunization during which immune lymphocytes could be stimulated to produce MIF. This capacity was present for about 2 wk and then disappeared which was in sharp contrast to antibody titers that persisted for several months. Booster injections were not given to the rabbits in an attempt to restimulate lymphocyte function or to test immunologic memory for the LPS-II antigen.

However, we are uncertain which subpopulation of immune lymphocytes is responsible for this response. The general assumption had been that lymphokines, such as MIF, were released by specific antigenic stimulation of immune T lymphocytes (31). The direct MIF assay does not differentiate the reactive lymphocyte populations in the respiratory cell suspensions. For the indirect MIF assay, nonadherent column-fractionated lymphocytes from the respiratory tract and spleen were used for in vitro cultures, but the subpopulation identification was not determined in our studies. Previously, such column-separated lymphocytes from guinea pig lymph node cell suspensions have been identified as predominantly T lymphocytes (20). However, it is noteworthy that tuberculin-purified protein derivative and endotoxin lipopolysaccharide, prepared from *E. coli* and *Serratia marcescens,* have been shown to stimulate both guinea pig B and T lymphocytes to produce MIF (32).

The importance of immune lymphocytes in the coordinated host defense of the lung might be to modulate or enhance the activities of pulmonary macrophages through their production of lymphokines such as MIF. However, the mechanism of lymphocyte-macrophage interaction and the actual effect of MIF on this interaction remains uncertain. Information about the effect of MIF on macrophage function has been obtained principally from peritoneal macrophage systems. Rabbit peritoneal macrophages showed better adherence and ameboid activity after prolonged exposure to supernatant fluids obtained from immune lymph node cell cultures (33). Likewise, guinea pig macrophages showed improved activity in several parameters, including phagocytosis, after exposure to MIF (34). Guinea pig macrophage bacteriostasis against *L. monocytogenes* was enhanced after prior incubation of macrophages with lymphocytes, obtained from lymph nodes of orthochlorobenzoyl bovine gamma globulin-immunized guinea pigs, and with the immunizing antigen (35). Yet, exposure of peritoneal macrophages to lymphocyte culture media possessing a high degree of MIF activity had no effect on macrophage bacterial capacity against the intracellular organism *L. monocytogenes* (36).

However, peritoneal and alveolar macrophages are sufficiently different cell types (37) that generalizations should be avoided. Alveolar macrophages were capable of responding to MIF in this rabbit system, contrary to reported findings in guinea pigs (38). Although lymphocytes accounted for only 7% of respiratory cells, it has been shown (31) that as few as 1% sensitized lymphocytes are sufficient to inhibit the migration of a population of normal unsensitized macrophages. There is ample evidence (3–7) that a variety of infectious agents can produce cellular immunity or delayed hypersensitivity in the respiratory tract. The biological significance of these cellular events will require further studies which utilize cells indigenous to the lungs.

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