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A mouse model for investigating the molecular pathogenesis of adenovirus pneumonia

(adenovirus early genes/cytokines/cytotoxic T cells/nude mice)

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ABSTRACT **Intranasal inoculation of type 5 adenovirus (Ad5) produced pneumonia in mice even though the virus did not replicate. To induce the pneumonia, however, a large viral infectious dose was required—i.e., 10^{10} plaque-forming units. Four strains of inbred mouse were studied (C57BL/6N, C57BL/10ScN, CBA/N, and C3H/N): all showed similar inflammatory responses, although the greatest infiltration occurred in the C57BL/6N mice. The pathological response to Ad5 infection resembled that previously described in cotton rats: it consisted of overlapping early and late phases, and the infiltration contained primarily lymphocytes and monocytes/macrophages with a scattering of polymorphonuclear leukocytes. The prominent early phase and the presence of polymorphonuclear leukocytes suggested that induction of cytokines may play an important role in the pathogenesis of this pneumonia. Assays showed the appearance of tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), and IL-6 in the infected mouse lungs concomitant with the developing early-phase infiltration. Only IL-6 was found in the peripheral blood. IL-6 reached maximum titers 6-24 hr after infection, whereas maximum levels of TNF-α and IL-1 were attained 2-3 days after infection. Specific RNAs for each of these cytokines were demonstrated in the infected lungs. To test the hypothesis that a cytotoxic T-cell response was responsible for the second phase, which primarily consisted of a perivascular and peribronchial infiltration of lymphocytes, Ad5 was used to infect C57BL/10ScN *Nu/Nu* and parent mice. The nude mice showed a normal early-phase response, but essentially no peribronchial and only minimal perivascular infiltrations occurred.**

Intranasal inoculation of type 5 adenovirus (Ad5) into the cotton rat *Sigmodon hispidus* initiates the development of a pneumonia that closely resembles that produced in humans (1, 2). This model was then used to investigate the viral genes required to produce the pathogenesis of the disease, which for these studies is termed "molecular pathogenesis." The results obtained, using mutants that contained defects in a 19-kDa glycoprotein, implied that the inflammatory response resulted from both the production of cytokines and the infiltration of cytotoxic T cells (3). Thus, it was hypothesized that the first phase of the pneumonia, which consists of a lymphocyte and monocyte/macrophage intraalveolar and interstitial infiltration as well as a scattering of polymorphonuclear leukocytes (PMNs), resulted from a local elaboration of cytokines. It was further hypothesized that the second phase, which is composed of a lymphocytic perivascular and bronchiolar infiltration, was due to a virus-specific cytotoxic T-cell response.

It was not readily possible to test these hypotheses in cotton rats since reagents are not available either to assay their cytokines or to identify the species of lymphocytes present in the pneumonic infiltration. It appeared possible to overcome these experimental barriers, however, when we demonstrated that H5ts125 (4), a conditionally lethal, temperature-sensitive mutant that is unable to replicate its viral DNA in the cotton rat's lungs, produces pneumonia the same as wild-type (wt) virus; therefore, only early viral gene functions are required to produce the disease (5). It was previously demonstrated that human adenoviruses cannot replicate in lungs of mice (unpublished data), but that early genes are expressed in cultured mouse cells (6). These findings suggested that if a large inoculum of Ad5 were used to infect mice intranasally, in order to infect a sufficient number of bronchial epithelial cells, a pneumonia would be produced similar to that seen in *S. hispidus* cotton rats (1, 2). It is the purpose of this communication to demonstrate that a large intranasal inoculum of Ad5 does induce a pneumonia similar to that produced in cotton rats and resembling that found in humans.

MATERIALS AND METHODS

Cell Cultures. Monolayer cultures of KB cells (4) and A549 cells (1) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) calf serum, as described (3, 4).

Viruses and Viral Assay Methods. Ad5 wt was used throughout this study. Stocks of wt virus were propagated in KB cell monolayers. Plaque assays were used to determine the quantity of infectious viral particles in viral stocks (7), and fluorescent focus assays were used to quantitate the virus in lung homogenates (8). A549 cells were used for both the plaque and the fluorescent focus assays (3, 5).

Animal Studies. Inbred strains of C57BL/6N, C57BL/10ScN, CBA/N, and C3H/N 3- to 4-week-old mice were used in these initial studies because of the ready availability of inbred congenic strains. C57BL/10ScN Nu/Nu mice were also used. All of the animals were obtained from the Veterinary Resources Branch, Division of Research Services, National Institutes of Health, Bethesda, MD. Mice were infected intranasally with 0.1 ml of virus diluted to contain 10^{11} plaque-forming units (pfu)/ml while under light inhalation methoxyflurane anesthesia. At the times indicated mice

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Abbreviations: Ad5, adenovirus type 5; wt, wild type; IL, interleukin; TNF, tumor necrosis factor; pfu, plaque-forming unit; PMN, polymorphonuclear leukocyte.

were sacrificed, and lungs were prepared for histologic examination and viral infectivity assays as described (3, 5).

Cytokine Bioactivity Assays. Lungs were rapidly removed and frozen immediately in liquid nitrogen until homogenized in 5 vol of 155 mM NaCl with a Polytron homogenizer at 4°C. A cell-free homogenate was obtained by two successive centrifugation cycles at 4°C for 30 min each: 3200 × *g* followed by 18,000 × *g*. The cell-free supernatant was sterilized by filtration and stored at −70°C. Blood was obtained by cardiac puncture, cells were removed from the heparinized blood by centrifugation, and the plasma was stored at −70°C.

Tumor necrosis factor type α (TNF-α) in plasma and lung supernatant fluids was assayed in triplicate by the WEHI 164 clone 13 fibroblast cytotoxicity bioassay previously described (9, 10). A TNF-α-specific antiserum neutralized all activity. Recombinant human TNF-α was used as a standard. Levels of interleukin (IL)-1α/β were determined by a competitive receptor–ligand binding assay to plasma membranes of the thymoma cell line EL-4 (10, 11). Competition was done with ^{125}I-labeled mouse IL-1α. IL-6 content of lungs and blood was assayed by quantitating the proliferation of an IL-6-dependent murine hybridoma cell line B.9 (10, 12). Neutralizing monoclonal antibody to murine IL-6 (generously provided by J. Van Smick, Brussels) was used to confirm that the proliferative response of the B.9 cells was due to murine IL-6.

Cytokine RNA Assay. Total cellular RNA was extracted in a single step from frozen lungs of Ad5-infected mice in 5 vol of guanidinium isothiocyanate, acid phenol extraction (RNAazol, Çinna Biotech, Friendswood, TX). One microgram of total cellular RNA was transcribed for 60 min at 37°C with 200 units of Moloney murine leukemia virus reverse transcriptase and 0.5 μg of oligo(dT) as primer. Specific cDNA fragments for murine IL-1α, IL-1β, and TNF-α were hybridized with the appropriate 20-base-pair oligonucleotide primers (1 μM each) and amplified with 0.5 unit of *Thermus aquaticus* DNA polymerase during 30 cycles. Each amplification cycle included denaturation at 92°C, reannealing of primer and fragments at 50°C, and primer extension at 72°C. Ten microliters of the 100-μl reaction mixture was fractionated on a 1% agarose gel. Samples were electrophoresed at 50 V for 2 hr and stained with ethidium bromide.

Semiquantitative Scoring of Pneumonia. To compare the degree of lung pathology that Ad5 wt virus produced in the different mouse strains at different intervals after infection, histological sections were examined without knowledge of the mouse strain or the time of sacrifice (i.e., scored blind). The number of lobes involved was counted, and the extent of alveolar, peribronchial, and perivascular inflammatory responses was graded on a scale of 0–4 (5). Data are expressed as mean scores from five or six mice.

RESULTS

Production of Ad Pneumonia in Mice. Four different inbred strains of mice were selected to ascertain whether Ad5 can produce pneumonia in mice even though it does not replicate in these animals (ref. 6; unpublished data). Inbred strains of CBA/N, C3H/N, C57BL/6N, and C57BL/10ScN were selected for these initial experiments because a number of congenic strains have been derived from each of the parent strains. When $10^{8.0}$ pfu per mouse was inoculated intranasally into CBA/N mice, a quantity of virus that produced pneumonia and multiplied in lungs of cotton rats (2, 3), pneumonia was not observed histologically and viral replication was not detectable. However, when 10^{10} pfu was inoculated, pneumonia developed, although viral replication still could not be detected. Similar experiments were done with the other three strains of mice listed above: the extent of pneumonias produced was compared, and infectivity assays were carried out on lung homogenates. Virus did not multiply in the lungs of any of these mouse strains (e.g., in C57BL/6N; Fig. 1). Nevertheless, pneumonia was produced in all four strains, probably because the large viral inoculum used yielded an initial viral titer in lung cells comparable to that attained in cotton rats by viral replication (3, 5). It is important to note that the greatest inflammatory response was produced in lungs of C57BL/6N mice (an overall score of 3 on a scale of 0–4). The semiquantitative scores of the overall inflammatory response obtained in the other mouse strains were as follows: CBA/N, 2.5; C3H/N, 2.0; C57BL/10ScN, 1.5.

Pathology of Ad5 Pneumonia and the Course of Its Development in C57BL/6N Mice. In C57BL/6N mice, as well as in the other strains studied, the inflammatory response after infection with 10^{10} pfu consisted of two phases, which were similar to those observed in the development of pneumonia in *S. hispidus* after intranasal infection with Ad5 (refs. 2 and 3; unpublished data). The earlier phase consisted of thickening of alveolar septa and septal infiltration of monocytes/macrophages and lymphocytes, intraalveolar infiltration with similar cells, plus a scattering of PMNs, perivascular infiltration with lymphocytes, and minimal peribronchial lymphocytic infiltration (Fig. 2). The macrophages lining the alveolar walls were large, granular, and appeared activated but did not show the cytopathology found in cotton rats (3). This early phase began 1–2 days after infection, was most prominent by day 3 or 4, and was still observed on day 5. The second phase consisted of very prominent lymphocytic perivascular and peribronchial infiltrations (Fig. 2 *C* and *D*), which attained their maximum 5–7 days after infection (Fig. 3). Clearly these two phases overlapped (Figs. 2 and 3). The pathologic process in mice differs somewhat from that in cotton rats in that the perivascular infiltration appeared earlier and was much more prominent in mice; and cytopathic changes in the mouse bronchial epithelial cells, which are the host cells in which Ad5 replication is detected in cotton rats (2, 3), were minimal except for minor clefting.

Cytokine Response to Ad5 Intranasal Infection. The early inflammatory response to Ad5 infection, and particularly the intraalveolar appearance of PMNs, suggested that cytokines may play a role in producing this infiltration. Accordingly, TNF-α, IL-1, and IL-6 were assayed in infected lung homogenates and plasma at 2-day intervals beginning on the

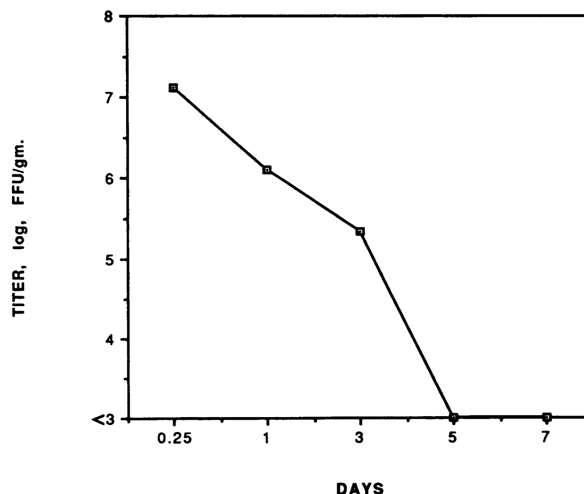
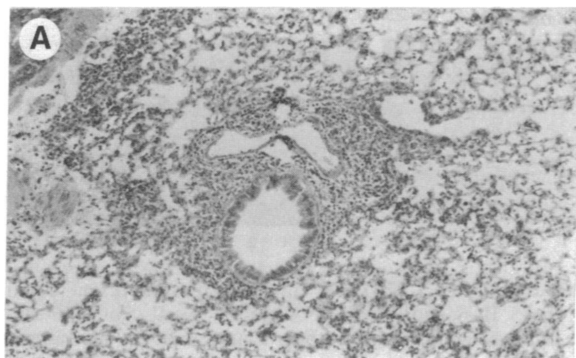
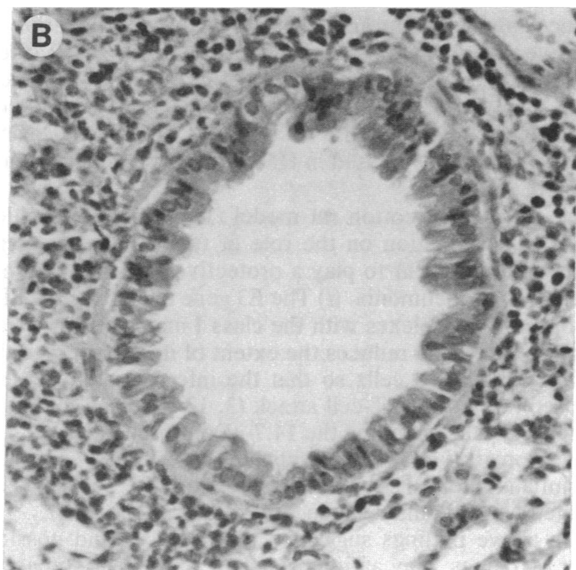


FIG. 1. Multiplication of Ad5 wt virus in lungs of C57BL/6N mice. Animals were infected intranasally with 10^{10} pfu. Mice were sacrificed at the indicated times after infection; infectivity titers were determined by a fluorescent focus assay on 10% (wt/wt) lung homogenates, and geometric mean titers of six animals per group were calculated and plotted. FFU, fluorescent focus units.





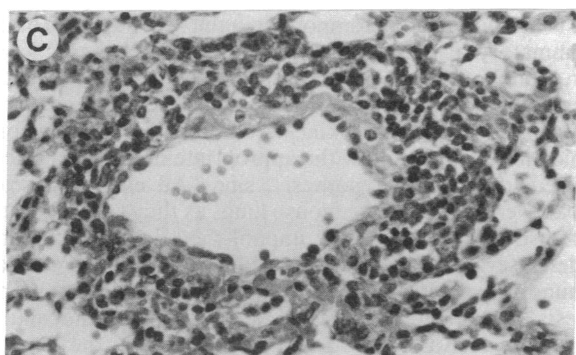


FIG. 2. Pathology of lungs from C57BL/6N mice 5 days after intranasal infection with 10^{10} pfu of Ad5 wt virus. (*A*, 45×; *B* and *C*, 110×.)

first day after infection. Similar specimens from uninfected controls were also assayed. The data from three experiments, summarized in Table 1, clearly show the appearance of all three cytokines in the infected lungs, but only IL-6 appeared in the peripheral blood of the infected mice. The kinetics of the cytokine responses differed, however. TNF-α reached maximum levels 2–3 days after infection and then declined rapidly; IL-1 also attained its maximum levels 2–3 days after infection but maintained these levels for the entire experimental period; in contrast, IL-6 gained its highest level by 1 day after infection and had declined significantly by the 3rd day. Indeed, when assayed only 6 hr after infection, IL-6 had reached a level of 378 units/ml, whereas TNF-α was <5 units/ml (a single experiment). Polymerase chain reaction data from one experiment, shown in Fig. 4, confirm the

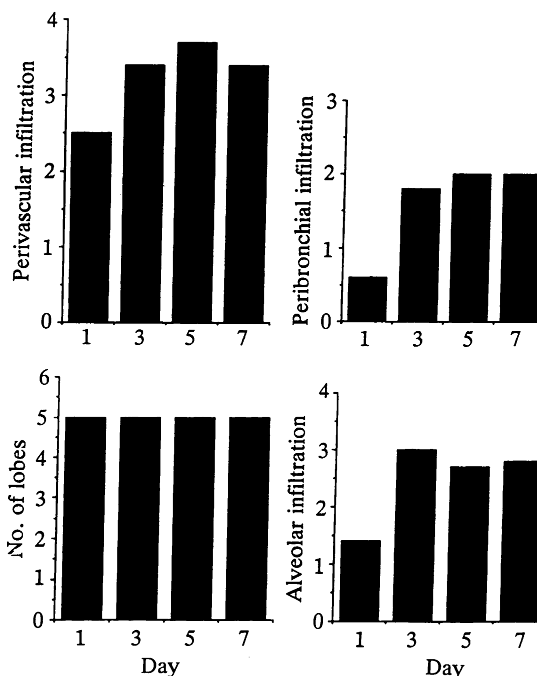


FIG. 3. Development of pneumonia after intranasal inoculation of 10^{10} pfu of Ad5 wt virus into groups of six C57BL/6N mice. The histological features of the slide were scored on a scale of 0–4 without knowledge of the day of inoculation; 0–5 lobes were scored. Each score is the mean of that determined with histological sections from groups of six animals.

cytokine production by demonstrating the induction of IL-1α, IL-1β, IL-6, and TNF RNAs in Ad5-infected lungs; IL-6 RNA induction was the most rapid.

Comparison of Inflammatory Responses in C57BL/10ScN Parent and Nu/Nu Mice. Earlier studies showed that Ad E3 mutations, which affected a gene encoding a 19-kDa glycoprotein, increased expression of class I major histocompatibility complex antigens on the surfaces of infected cells and markedly increased the extent of pneumonia produced by intranasal infection of cotton rats (3). These data suggested

Table 1. Elaboration of TNF-α, IL-1, and IL-6 after Ad5 infection of mouse lungs

Day after infection	TNF-α, units/ml		IL-1, ng/ml		IL-6, units/ml	
	Plasma	Lungs	Plasma	Lungs	Plasma	Lungs
0*	<2	<2	<2.5	<2.5	<5	<5
1	<2	18	<2.5	22	34	166
3	<2	26	<2.5	72	9	143
5	<2	5	<2.5	65	12	87
7	<2	3	<2.5	57	9	29

C57BL/6N mice were infected intranasally with 10^{10} pfu of Ad5 except those sacrificed at time 0 (see *). Values represent the geometric means of results from three experiments with samples pooled from four animals per group for each experiment. In an additional experiment, lung homogenates from animals infected 6 hr before sacrifice contained 378 units of IL-6 per ml and <5 units of TNF-α per ml. TNF-α bioactivity was determined with the WEHI clone 13 cytotoxicity assay; all activity could be neutralized with a rabbit polyclonal antiserum from animals immunized with recombinant murine TNF-α. TNF-α and IL-6 values represent units/ml of a 20% (wt/vol) homogenate. IL-1α/β was determined by a competitive receptor–ligand assay to membranes of thymoma cell line EL-4. IL-6 was determined by the B.9 hybridoma cell proliferation assay; a rat anti-mouse IL-6 monoclonal antibody neutralized all activity.

*Results from a single experiment in which at time 0 control mice were inoculated intranasally with physiologic saline.

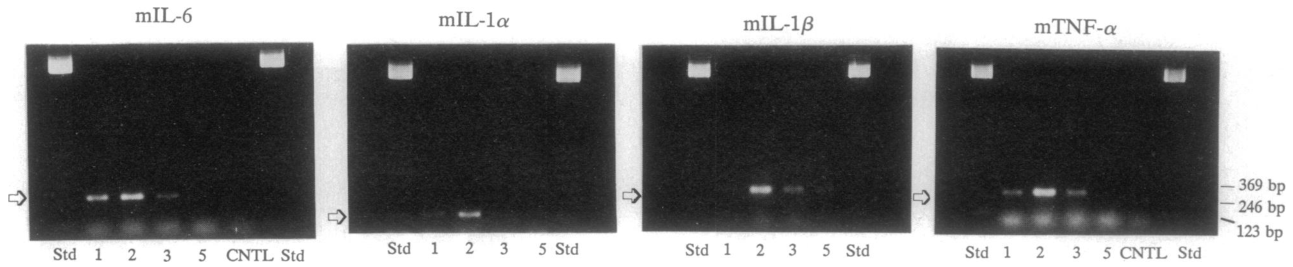


FIG. 4. Polymerase chain reaction assay for cytokine RNAs in lungs of Ad5-infected C57BL/6N mice. Arrows represent ethidium bromide-stained, amplified cDNA derived from the respective murine (m) IL-1α, IL-1β, IL-6, and TNF-α RNA species. No IL-6 or TNF-α RNA was detected in lungs of uninfected mice (CNTL). Std, DNA length standards; bp, base pairs. Numbers below lanes indicate days after Ad5 infection.

that cytotoxic T cells play an important role in the production of Ad pneumonia in the cotton rat model, and that the markedly increased pneumonic infiltration produced by the mutants unable to express the E3 19-kDa glycoprotein resulted from an increased response of cytotoxic T cells to the infection. To test this hypothesis, C57BL/10ScN parent and congenic Nu/Nu mice were infected intranasally with 10^{10} pfu of Ad5 virus, and the pathological responses to infection were studied. After day 3, the inflammatory response was markedly decreased in the nude mice (Fig. 5); this decreased pathology was particularly dramatic after day 5. The decreased peribronchial and bronchial wall lymphocytic infiltration as well as the decreased perivascular infiltration were especially striking. These data support the notion that a major part of the pathogenic process results from a strong cytotoxic T-cell response to Ad5-infected cells.

DISCUSSION

The data described above indicate that the mouse can serve as a useful animal model to investigate the molecular pathogenesis of Ad pneumonia. It is a unique animal model since viral replication does not precede or accompany the pathogenic process, whereas in all other animal models previously used to study viral infections complete viral multiplication is a central component. The suggestion that adenoviruses might

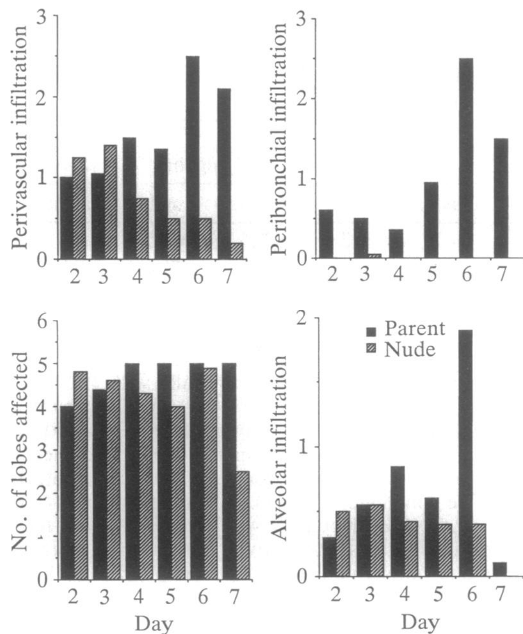


FIG. 5. Development of pneumonia in parent and congenic Nu/Nu C57BL/10ScN mice after intranasal inoculation of 10^{10} pfu of Ad5 wt virus. Pathology was scored on groups of six mice as noted in Fig. 3.

produce disease in an animal in which viral replication is defective emerged from the finding that infection of cotton rats with a conditionally lethal, temperature-sensitive mutant (H5ts125) in the E2A DNA-binding protein gene (4) produced pneumonia similar to Ad5 wt virus (5). Thus, only early genes, which are expressed in H5ts125-infected cells (4), are required.

The *S. hispidus* cotton rat model (1, 2) has also yielded important information on the role of two other early gene functions that appear to play a protective role in the pathogenesis of Ad pneumonia. (*i*) The E3 gene encoding a 19-kDa glycoprotein complexes with the class I major histocompatibility complex and reduces the extent of its transport to the surface of infected cells so that the infected cells are less exposed to cytotoxic T-cell attack (3, 13–15). (*ii*) Mutations in the E3B gene encoding the 14.7-kDa protein suppress the expression of a factor that affects the appearance of PMNs in the inflammatory response to infection (3) and protects infected cells in culture from TNF-induced lysis (16).

The above findings suggested that TNF-α, and possibly other cytokines, play an important role in the early phase of the pathogenic process, and that infiltration of cytotoxic T cells is a major cellular component in the second phase of the inflammatory response to infection. These hypotheses, however, could not be tested in the cotton rat because the essential reagents to identify the species of lymphocytes or to detect and quantitate cytokines are not available. This communication describes how the appropriate mouse strains can be used to test this hypothesis, since Ad early genes are probably expressed in the mouse lungs as they are in cultures of mouse fibroblasts (ref. 6; unpublished data). The animal model described offers the opportunity to study the cellular, immunological, and molecular factors effecting the pathogenesis of Ad pneumonia. Thus, after intranasal inoculation of 10^{10} pfu of Ad5, TNF-α and IL-1 appear in the infected mouse lungs in amounts that parallel the extent of the early phase of the inflammatory cellular infiltration (Table 1), whereas none is detectable in the blood. However, IL-6 appears earlier, declines rapidly, and is present in both the lungs and blood, which indicates that in Ad5 infection of mouse lungs IL-6 is induced through a TNF-independent pathway, in contrast to that involved in Gram-negative bacterial infections (17). Infection of parental and nude/nude C57BL/10ScN mice presented evidence indicating that the perivascular and peribronchial lymphocytic infiltrations consist of cytotoxic T cells and probably T_4 helper cells, as suggested in earlier studies (3), since such infiltration is markedly reduced, and even absent in the latter phase of infection in nude mice (Fig. 5).

Infections of cell cultures with a variety of both RNA- and DNA-containing viruses induce the elaboration of TNF, IL-1, and IL-6 (18–22). Furthermore, lymphocytic choriomeningitis virus (LCMV) and vesicular stomatitis virus (VSV) infections of the central nervous system of mice induce IL-6 production (22). It is striking, however, that

LCMV induces elaboration of much lower levels of this cytokine in nude mice, whereas VSV affects levels very similar to that induced in normal mice (23). These data add further evidence indicating that LCM is caused by an induced T-cell response and that IL-6 production also requires a T-cell dependent pathway (23). It has also been reported (24) that TNF-α and TNF-β (lymphotoxin) inhibit replication of RNA- (VSV and encephalomyocarditis virus) and DNA-containing viruses (Ad2 and type 2 herpes simplex virus) and that TNF and interferon are synergistic (24). Moreover, TNF induces β-interferon, which explains this antiviral effect (27). However, antiviral interferon activity could not be detected in the Ad5-infected mouse lungs from our experiments (E. H. Havel, Trudeau Institute, personal communication). It is also unlikely that the inability of Ad5 to multiply in mouse lungs is due to death of infected cells owing to the TNF-α elaborated (24), since TNF only lyses Ad-infected cells when the infecting virus contains a mutation in the E3 14.7-kDa protein gene (16). Furthermore, it must be noted that lysis and marked shedding of the infected bronchiolar epithelial cells is not apparent in either the cotton rat (1–3) or this mouse model (see Fig. 2).

Viral pneumonias in humans and in animal models primarily consist of a lymphocyte–monocyte/macrophage infiltration just as described for Ad5 pneumonia in cotton rats (1–3, 5) and in this mouse model. Cytokines have been assigned an important role in recruitment of these cells into the infected lung (25). IL-1, IL-6, and TNF-α, as well as interferons, interact in a cytokine network that produces adherence of lymphocytes to endothelial cells to permit their transport into the lungs, activates resident cells (i.e., lymphocytes, macrophages, and fibroblasts), induces further production of lymphokines, and effects additional recruitment of lymphocytes from blood (25, 26). Identification of the early gene(s) expressed and the role(s) they play in the induction and elaboration of cytokines in the pulmonary, inflammatory response described in this animal virus model of Ad5 pneumonia are not yet known. In addition, studies should be initiated to determine which early viral protein(s) is processed and displayed on the surfaces of infected cells to serve as a signal for proliferation of the cytotoxic T cells that represent a prominent component of the inflammatory response to the Ad5 pulmonary infection.

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