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# Role of early region 3 (E3) in pathogenesis of adenovirus disease

**(E3 gene products/class I major histocompatibility complex antigens/19-kDa glycoprotein/cytotoxic T cells)**

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ABSTRACT **The cotton rat *Sigmodon hispidus* has provided an animal model of adenovirus pneumonia that permits investigation of the viral gene products required to produce the disease and the molecular mechanisms effecting the damage. This study was carried out to test the hypothesis that early region 3 (E3) of the adenovirus genome plays a critical role in pathogenesis of the virus's disease process even though none of its gene products are essential for its replication. Mutants whose E3 region is largely deleted (i.e., H2dl801 and H5dl327) replicated like wild-type virus in the cotton rats' lungs, but the lymphocyte and macrophage/monocyte inflammatory response was markedly increased. Viruses containing mutations that ablated production of the 19-kDa glycoprotein had the same effect as H2dl801 and H5dl327. However, mutants with deletions in the other E3 open reading frames, some of which encode known proteins, did not differ from wild-type virus in their pathogenic properties. The 19-kDa glycoprotein markedly reduces expression of the class I major histocompatibility complex antigens on the surface of infected cells. A complete correlation was found between those mutants that had increased pathogenic effects and those that lost the ability to reduce transport of the class I major histocompatibility complex antigens to surface of infected cells (i.e., all mutants unable to express the 19-kDa glycoprotein). H5sub304, which has a deletion between 83.2 and 85.1 map units in the E3B region and expresses the 19-kDa glycoprotein, did not increase the extent of pneumonia but qualitatively changed the inflammatory response in that increased numbers of polymorphonuclear leukocytes accumulated, often in small foci.**

One utility of the modern techniques of virology and molecular biology should be to reveal the basic molecular mechanisms by which viruses such as adenoviruses produce disease. The discovery that intranasal inoculation of type 5 adenovirus (Ad5) into cotton rats results in development of a pneumonia that pathologically simulates that produced in humans (1) provided the opportunity to determine the viral gene functions required to produce the pneumonia and the molecular mechanisms by which the virus induced the disease. Extensive studies have been carried out on the replication of adenovirus in the lungs of the *Sigmodon hispidus* species of cotton rats and on the relationship of viral multiplication to the development of pneumonia (2, 3). Productive viral replication was only detected in the epithelial cells of the bronchi and bronchioles of the lung and the nasal mucosa. Onset of viral multiplication, which reached maximum titers 2–4 days after infection (depending upon the size of the inoculum), was soon followed by progressively increasing peribronchial, perivascular, and alveolar septal infiltration of lymphocytes and monocyte/macrophages and finally by lymphocytic infiltration of the basal bronchiolar wall into the epithelium; scattered polymorphonuclear leukocytes (PMN) were also present (2, 3). The maximum pathology was attained 5–7 days after infection, which was also dependent upon the viral inoculum. The use of conditionally lethal, temperature-sensitive mutants [e.g., H5ts125 (4)] unable to replicate their DNA at the cotton rat's normal body temperature, about 39.2°C, led to unexpected results: the cellular inflammatory response was the same as in wild-type (Wt) virus-infected lungs, although quantitatively not as extensive. These data indicate that only early gene products appear necessary to induce the inflammatory response to viral infection. It was further demonstrated that the E1B 58-kDa protein, which is required to shut off host protein synthesis during adenovirus productive infection (5, 6), is also essential for producing maximum viral pneumonia (3).

Early region 3 (E3) has been termed a "nonessential" region since naturally occurring mutants or hybrid viruses in which almost the entire region is deleted still replicate like Wt virus in cultured cells (7). It seemed unlikely that almost 10% of the genome would have survived in evolution if its encoded genes did not play a significant role in the virus's life cycle. Therefore, extensive studies were done to determine whether the E3 region might play a critical role in pathogenesis. It is the objective of this communication to report the results of that investigation. Data will be presented to show that gene products of the E3 region do in fact play a strategic role in viral pathogenesis. However, their role appears to ameliorate the response and thus protect the host from extensive disease, as well as to protect the virus from eradication. It will be shown that deletion of an E3-encoded 19-kDa glycoprotein (gp19kDa) (8), which reduces expression of the class I major histocompatibility (MHC) antigens on the surface of cultured infected cells (9–11), results in a virus that produces a significantly more extensive pneumonia than its parental virus. Moreover, deletion of the 3′ portion of E3, which encodes the 10.4-, 7.5-, 14.5-, and 14.7-kDa proteins (12), results in a mutant [e.g., H5sub304 (13)] that does not increase the extent of the pneumonia but affects the nature of the inflammatory response.

## MATERIALS AND METHODS

**Cell Cultures.** Monolayer cultures of KB (4) and A549 (kindly supplied by E. J. Dubovi, Cornell University, Ithaca, NY) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum.

**Viruses and Assay Methods.** Types 2 and 5 Wt viruses previously described (14) and an Ad5 containing a portion of the E3 region of Ad2, H5/2rec700 (15), served as the Wt virus controls for the E3 mutants constructed in this region. The

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Abbreviations: MHC, major histocompatibility complex; Wt, wild type; Ad2, Ad4, Ad5, and Ad7, adenovirus types 2, 4, 5, and 7; PMN, polymorphonuclear leukocytes.

characteristics of the E3 mutants employed are shown in Table 1. Mutant H2dl801 (16) and mutants H5dl327 and H5sub304 (13) were generously provided by G. Ketner (Johns Hopkins University) and T. Shenk (Princeton University), respectively. The deletion mutants (Table 1) were derived from H5/2rec700, as described (15, 17).

Viral infectivity was determined by indirect immunofluorescence assays on KB cells using polyclonal rabbit antiserum prepared with intact virus (18) or plaque assays (19) with A549 cells.

**Animal Model.** Cotton rats, an inbred strain of *S. hispidus*, were obtained from the Veterinary Resources Branch, Division of Research Services, National Institutes of Health. Animals of various ages, 3–6 weeks, were used since preliminary experiments indicated that the response to adenovirus infection did not significantly vary with the age of the animals. In a single experiment, cotton rats of the same age were used. Animals were infected intranasally under light inhalation methoxyflurane anesthesia. Upon sacrifice of the animals, lungs from one-half of the animals were inflated with 10% neutral buffered formalin, fixed in formalin for at least 24 hr, and embedded in paraffin for sectioning. Histologic sections were stained with hematoxylin and eosin. Control lung sections were obtained from mock-infected animals. To determine viral titers, 10% homogenates in Hanks' balanced salt solution were prepared with lungs from each of the other animals sacrificed at indicated times.

**Quantitation of Class I MHC Antigens.** Staphylococcus protein A-Sepharose-purified monoclonal antibodies (W6/32) to human class I MHC (reacted with HLA-A and -B; obtained from American Type Culture Collection) were conjugated to fluorescein isothiocyanate. Suspensions of uninfected and adenovirus-infected KB cells were washed twice with phosphate-buffered saline, resuspended in phosphate-buffered saline to a concentration of $2 \times 10^6$ in a 1:50 dilution of antiserum, and held at 4°C for 30 min. The cells were then washed, and the expression of the class I MHC antigens was determined by flow-cell cytometry (Becton Dickinson FACS IV).

## RESULTS

**Pathogenic Effect of Naturally Occurring E3 Deletions.** A number of mutants containing deletions of various extents in the E3 region have been isolated from stocks of several types of so-called Wt adenoviruses, and in each instance the virus was not defective in its replication in cell cultures (20). Two such mutants, H2dl801 (16) and H5dl327, were used to determine whether they also replicated like their corresponding Wt virus in the lungs of cotton rats and whether their pathological effects differed from Wt virus in the lungs of the infected animals. The results of one such experiment comparing H2dl801 and Wt Ad2 viruses (Fig. 1) clearly showed that H2dl801 replicated to the same extent (and was cleared as rapidly) as Wt virus in cotton rat lungs, despite the extensive E3 deletion (between 78.5 and 83.5 map units; i.e., 747–2684 base pairs; Table 1).

The replication characteristics of the two viruses were similar; however, the pathologic cellular response of the cotton rat lung to infection with H2dl801 was significantly greater than that to Ad2 Wt virus at every stage of the disease. Fig. 2 illustrates the consistent differences in the extent of the inflammatory responses to the two viruses. The fundamental cellular responses to the two viruses were not basically different—i.e., peribronchiolar monocyte infiltration, interalveolar cellular exudation and interstitial thickening, damage to but not lysis of bronchiolar epithelium, increased peribronchiolar lymphocytic infiltration and bronchiolar wall lymphocytic invasion, and perivascular lymphocytic infiltration. However, the time at which the inflammation appeared

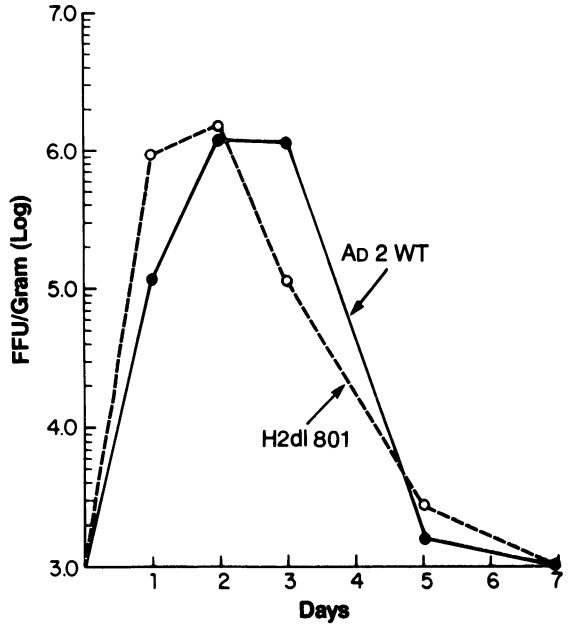


FIG. 1. Replication of Wt Ad2 and H2dl801 in cotton rat lungs. Each animal was infected intranasally with $10^8$ plaque-forming units of virus; at the indicated times five animals per group were sacrificed, and a 10% homogenate of each lung was prepared in Hanks' balanced salt solution. Infectivity titers were determined by fluorescent focus assays, and they are expressed as geometric means of the five titers. FFU, focus-forming units.

was earlier in H2dl801-infected lungs, and the extent of the peribronchiolar, perivascular, and interalveolar lymphocyte and macrophage/monocyte infiltration was considerably greater than that in the lungs infected with Wt virus. In addition, the early appearance of small foci of PMNs was more extensive, and the later invasion of lymphocytes into the bronchial and bronchiolar walls and epithelial layers was much greater in the lungs of animals infected with H2dl801. H5dl327, which has an E3 deletion slightly larger than that of H2dl801 (Table 1) so that it cannot express the 14.7-kDa protein (W.S.M.W., unpublished data), also produced a significantly greater pulmonary infiltration than Ad5 Wt virus, and it too multiplied like its type-specific Wt virus.

**Identification of the E3 Gene Effecting Increased Pathogenesis.** To consider the mechanism of the unusual phenomenon by which a large genome deletion results in a virus that is more virulent than its related Wt virus, it was necessary to

Table 1. Characteristics of E3 mutants studied

| Mutant | DNA deleted | Gene products affected* (in kDa) |
|---|---|---|
| H5dl327 | 78.5–84.3 mu | gp19kDa, 14.7, 11.6, 10.4 (6.7, 7.5, 14.5) |
| H2dl801 | 747–2684 bp | gp19kDa, 11.6, 10.4 (3.6, 6.7, 7.5, 14.5) |
| H5/2dl703† | 1211–1249 bp | gp19kDa |
| H5/2dl704 | 1289–1441 bp | gp19kDa |
| H5/2dl706 | 1604–2238 bp | gp19kDa, 11.6, 10.4 (7.5) |
| H5/2dl754 | 1200–1254 bp | gp19kDa (6.7) |
| H5sub304 | 83.2–85.1 mu | 10.4, 14.7 (7.5, 14.5) |
| H5/2dl701 | 1028–1071 bp | (6.7) |
| H5/2dl712 | 1691–2122 bp | 11.6 |
| H5/2dl733 | 477–666 bp | (12.5) |

mu, Map units; bp, base pairs.
*Undetected, potential gene products of open reading frames are in parentheses.
†The mutated segment of the E3 region from Ad2 was inserted into the remainder of the Ad5 genome. The parent unmutated strain is H5/2rec700.

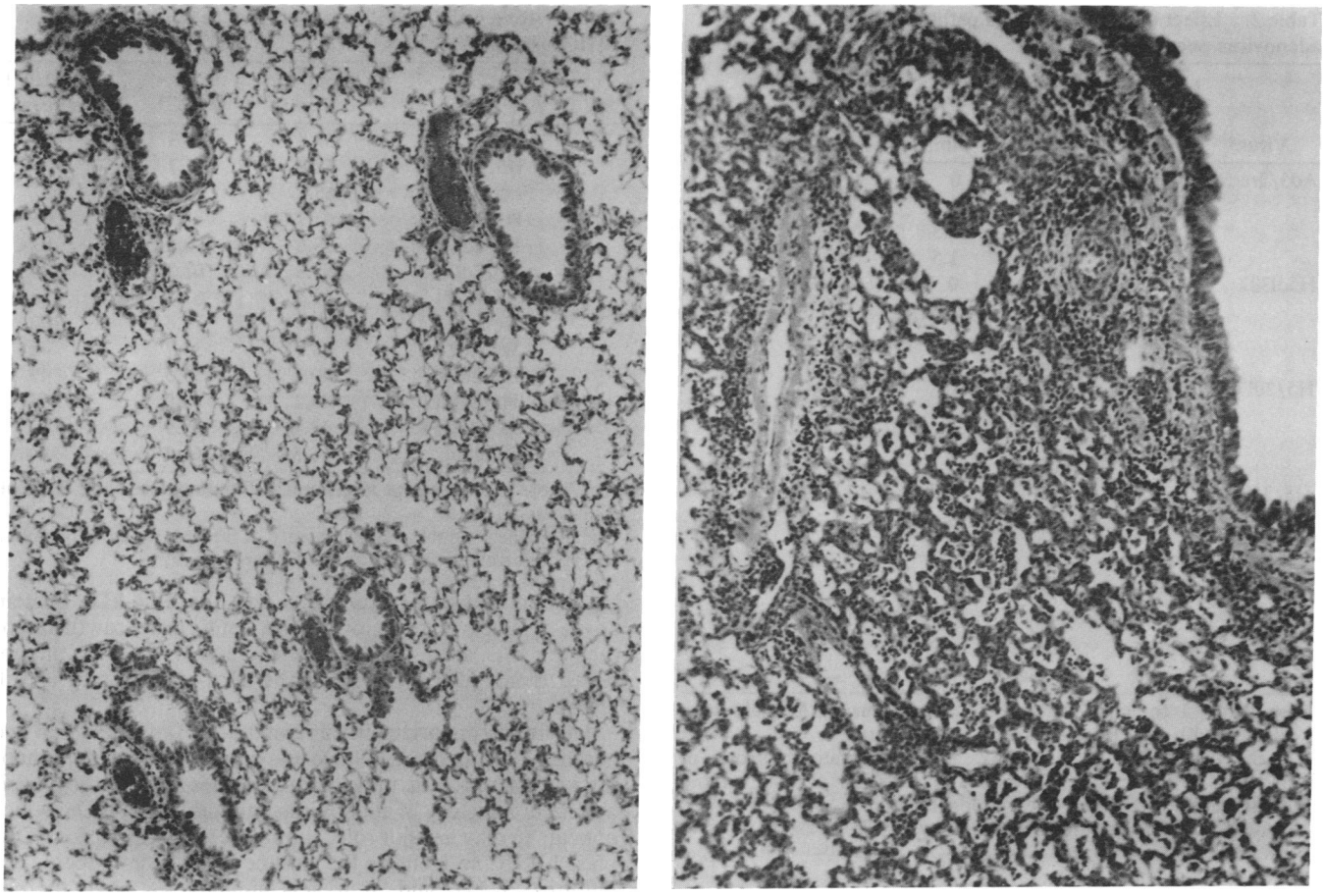

FIG. 2. Pathological response in lungs of cotton rats 3 days after intranasal inoculation of $10^8$ plaque-forming units of Wt Ad2 (*Left*) (average score of 1+) or H2dl801 (*Right*) (average score of 3+).

identify which gene(s) was responsible for this unusual phenomenon. The E3 genome contains nine open reading frames (12, 15), of which four gene products have been identified (8, 12, 21, 22). The mutants described in Table 1, each of which contained a single deletion affecting at least one open reading frame, were used to infect *S. hispidus* cotton rats to determine their pathogenic potential and replication characteristics as compared to its parental Wt virus. A semiquantitative analysis of the pulmonary response (Table 2) in a typical experiment indicated that a virus containing only a deletion in the gene encoding gp19kDa (Table 1) reproduced the increased pathogenicity manifested by H2dl801 (Fig. 2) and H5dl327. In contrast, mutants with deletions in the 6.7-kDa protein (H5/2dl701), the 11.6-kDa protein (H5/2dl712), and a putative 12.5-kDa protein (H5/2dl733), which has not yet been identified, produced pneumonia that was not different from the response to Wt virus (data not shown).

**E3 Mutations Affecting Expression of Class I MHC Antigens on the Surface of Infected Cells.** To determine whether the marked increase in pathogenicity produced by the mutations affecting the gp19kDa gene correlated with the ability of gp19kDa to reduce expression of the class I MHC antigens on the surface of infected cells, mutants used in the animal model experiments were studied to determine their effect on expression of class I MHC antigens on the cell surface. The data from two typical experiments using mutants abrogating production of functional gp19kDa or other E3 gene products (Table 3) clearly showed that only viruses containing deletions in the gp19kDa gene permitted expression of class I MHC antigens on the surface of uninfected cells in an amount approaching that on the surface of uninfected cells. Infection with viruses containing mutations in regions of the E3 genome encoding genes for other identified or unidentified proteins reduced expression of the class I MHC antigens to the same extent as Wt virus.

Infection with Ad2, Ad5, or Ad5/2 (rec 700) Wt viruses did not completely prevent transport of the class I MHC antigens to the cell surface (Table 3). It is likely that gp19kDa does not complex identically with all three of the class I MHC antigens. Indeed, it has been shown that gp19kDa binds more strongly to HLA-A2 than to HLA-B7 (23). It has also been demonstrated that in mouse cells transfected with either the $H\text{-}2K^b$ or the $H\text{-}2D^b$ class I MHC antigen gene, Ad2 infection only inhibited expression of the $H\text{-}2D^b$ antigen (24, 25).

**Effect of Deletion in the 3′ End of E3 on Pathogenesis.** H5sub304, a mutant isolated from a stock of Ad5 Wt virus (13), has a deletion and a substitution of a small piece of foreign DNA between 83.2 and 85.1 map units within the so-called E3B region (Table 1). Like the other E3 mutants, H5sub304 replicated like Wt virus in cell cultures and in the lungs of *S. hispidus*. In contrast with those mutants with defects in gp19kDa, H5sub304 did not produce a significantly greater general inflammatory infiltration in the cotton rats' lungs than did Wt virus. H5sub304-infected lungs, however, contained a significantly greater number of PMNs (Table 4) than did Ad5 Wt-infected lungs, and the PMNs were often in small foci. Unlike a response to a bacterial infection, however, the collections of PMNs were not associated with accumulations of fibrin or edema fluid.

## DISCUSSION

The E3 transcription unit of Ad2 and Ad5 produces nine mRNAs, which contain nine different open reading frames.

Table 2. Effect of E3 deletions on pathogenesis of adenovirus pneumonia

| Virus* | Day | Bronchioles | | Infiltration† | |
|---|---|---|---|---|---|
| | | % Positive | Epithelium† | Perivascular | Alveolar |
| Ad5/2rec700 | 1 | 7 | 0 | 0 | 0 |
| | 3 | 64 | 1 | 1 | 1 |
| | 5 | 99 | 2.5 | 1.5 | 1 |
| | 7 | 97 | 1.5 | 1 | 1 |
| H5dl327 | 1 | 92 | 0 | 1 | 1 |
| | 3 | 99 | 2 | 2 | 2 |
| | 5 | 100 | 3 | 3 | 2 |
| | 7 | 100‡ | 2 | 1.5 | 3 |
| H5/2dl703 | 1 | 66 | 0 | 0 | 1 |
| | 3 | 100 | 1 | 1 | 1 |
| | 5 | 100 | 3.5 | 3 | 3 |
| | 7 | 100§ | 2 | 3.75 | 2 |
| H5/2dl704 | 1 | 71 | 0 | 1 | 1 |
| | 3 | 100 | 2 | 2 | 3 |
| | 5 | 100 | 3.5 | 3 | 3 |
| | 7 | 100¶ | 2.5 | 2 | 2 |
| H5/2dl754 | 1 | 82 | 0 | 1 | 1 |
| | 3 | 100 | 2 | 2 | 2 |
| | 5 | 100 | 3.5 | 3 | 3 |
| | 7 | All dead | — | — | — |

*See Table 1 for characteristics of mutants used. Animals were infected with $10^{8.0}$ plaque-forming units of virus.
†Mean score of six animals. The extent of inflammatory response or epithelial cell damage was graded on a scale of 0–4 without identification of virus or interval postinfection.
‡Two of the six animals in group died by day 7.
§One of the animals died.
¶Two of the animals died.

Four unique gene products have been identified: gp19kDa and the 11.6-kDa, 10.4-kDa, and 14.7-kDa proteins (refs. 12, 21, and 22; A. E. Tollefson and W.S.M.W., unpublished data). Despite its relatively complex coding potential, the entire E3 region can be deleted without loss of the mutated virus's ability to replicate in cell cultures (7, 20); therefore, such mutants are commonly found in stocks of so-called Wt viruses (13, 16, 20). The E3 region is also not required for replication in the lungs of cotton rats, as shown in this communication, or of hamsters (26). However, functions of three of the E3 proteins have been demonstrated: the gp19kDa, a transmembrane protein, complexes noncovalently with the class I MHC antigens in the endoplasmic reticulum (27), inhibits their glycosylation, and reduces their transport to the surface of infected cells (9–11, 24); the 14.7-kDa protein protects infected cells in culture from lysis by tumor necrosis factor (28); and the 10.4-kDa protein down-regulates expression of the epidermal growth factor receptor (C. Carlin, A. E. Tollefson, and W.S.M.W., unpublished data). The function of the 11.6-kDa protein is still unknown.

Table 3. Role of E3 gp19kDa on cell surface expression of class I MHC antigens

| Exp. | Virus* | Relative cell size | FITC† | % MHC I antigens |
|---|---|---|---|---|
| 1 | None | 67.8 | 84.3 | 100 |
| | H5/2rec700 | 68.0 | 45.7 | 54 |
| | H5/2dl703 | 65.8 | 90.3 | 107 |
| | H5/2dl704 | 67.7 | 71.3 | 85 |
| | H5/2dl706 | 68.6 | 70.1 | 83 |
| | H5/2dl754 | 65.3 | 67.2 | 80 |
| | H5dl327 | 66.9 | 67.2 | 80 |
| 2 | None | 47.5 | 73.8 | 100 |
| | Wt Ad2 | 48.7 | 33.7 | 46 |
| | H5/2dl701 | 49.3 | 36.5 | 49 |
| | H5/2dl712 | 49.2 | 40.0 | 54 |
| | H5/2dl733 | 49.1 | 31.9 | 43 |
| | H2dl801 | 48.3 | 62.1 | 84 |

*See Table 1 for deletions in genomes of mutants used and the gene functions affected.
†Relative linear fluorescence intensity of fluorescein isothiocyanate (FITC), corrected for background fluorescence.

The data summarized in this communication imply that two of these E3 proteins, gp19kDa and one other protein, perhaps the 14.7-kDa protein, play important roles in the pathogenesis and natural history of adenovirus infections. gp19kDa, which appears to play a major role, suppresses the expression of the class I MHC antigens on the surface of Wt virus-infected cell cultures (9–11), and, if it acts similarly *in vivo*, thereby reduces cytotoxic T cells from attacking infected cells (29). Thus, in Wt virus infection, the functional gp19kDa decreases the lymphocytic inflammatory response. Moreover, this role of gp19kDa should also protect the virus within infected cells from eradication by adenovirus-specific cytotoxic T cells and probably accounts for the ability of these viruses to produce latent infections (30–32). It must be noted, however, that the pulmonary inflammatory response begins prior to the time that specific cytotoxic T cells would be expected to appear. These data suggest that the early lymphocyte and monocyte/macrophage infiltration may be due to a response to cytokines (e.g., interleukin 1 and tumor necrosis factor) released by activated macrophages and lymphocytes. The signal to initiate the process must originate from virus-infected cells displaying one or more viral proteins on their surfaces. One such viral protein may be the E1B 58-kDa protein since

Table 4. Role of gene products in the 3′ end of early region 3 (E3B) in pathogenesis

| Virus* | Day | % bronchioles affected† | Infiltration | | |
|---|---|---|---|---|---|
| | | | Lobes† | Degree‡ | PMNs |
| Wt Ad5 | 1 | 7.5 | 0 | 0 | 0 |
| | 3 | 62.0 | 4 | 1 | Small foci |
| | 5 | 100 | 5 | 2 | Rare |
| | 7 | 100 | 5 | 1 | Rare |
| H5sub304§ | 1 | 73 | 1 | 1 | Small foci |
| | 3 | 63 | 3 | 1 | Scattered |
| | 5 | 100 | 5 | 3 | Many scattered; many small foci |
| | 7 | 100 | 5 | 2 | Many scattered; many small foci |

*Each animal was infected with $10^{8.0}$ plaque-forming units of virus.
†Mean score of six animals.
‡The degree of infiltration was graded on a scale of 0–4. Mean score of six animals.
§The genome was deleted from 83.2–85.1 map units and a small fragment of salmon sperm DNA was inserted.

mutants that do not produce it show a markedly decreased inflammatory response, even though the other early gene products are made and infectious virus is replicated almost to Wt virus levels (H.S.G. and G.A.P., unpublished results). Since the E3 deletions that ablate production of gp19kDa markedly increase the early cellular infiltration as well as the lymphocytic invasion of the bronchial and bronchiolar walls and epithelial cell layers, the increased expression of the class I MHC antigens may itself increase the putative cytokine production or effect increased cell surface expression of the specific viral antigen(s) signals. It is also possible that gp19kDa has another function, not yet discovered, that is related to the putative cytokine production.

The increased number of PMNs in lungs of animals infected with viruses containing large E3 deletions or a mutant unable to express the 14.7-kDa protein (e.g., H5sub304, Table 4) is consistent with the hypothesis that the early inflammatory response to adenovirus infection results from liberation of cytokines. These latter findings support the notion that in Wt virus infections the 14.7-kDa protein may protect the host from either tumor necrosis factor excretion or tumor necrosis factor cellular responses. However, since H2dl801 also increases the numbers of PMNs even though the 14.7-kDa protein is made (28, 33), other E3 gene products must also effect this response. Since H5sub304 also lacks the genes for the 10.4-kDa protein, and for the putative 7.5-kDa and 14.5-kDa proteins (Table 1), one or more of these proteins may be involved in the PMN response.

Mutant H5/2dl712, whose genome contains a single E3 deletion that prevented expression of only the 11.6-kDa protein, did not affect the pathogenic response to pulmonary infection as compared with Wt virus. Moreover, H5/2dl712 reduced the cell surface expression of the class I MHC antigens to the same extent as the parental Wt virus (Table 3).

These findings not only shed light on the basic mechanisms by which one family of viruses, Adenoviridae, produce one of their pathogenic effects, but they also suggest that other viruses that primarily produce a monocytic cellular response to infection with or without extensive cellular lysis may also employ a similar mechanism to effect their pathogenic processes. Indeed, recently it has been shown that type 2 herpes simplex virus decreases expression of class I MHC antigens on the surface of infected cells (34). Moreover, although our studies are concerned with the mechanism of pathogenesis for a single virus and only one disease, the data presented also show clearly that regions of the genome unessential for replication of the virus may contain genes coding for proteins that have other critical functions in the biological roles of the virus. These findings support the contention that it is unlikely that evolution would permit a virus to maintain large segments of its limited genome without their encoded genes having an advantageous utility.

This proposed mechanism of pathogenesis assumes additional importance in view of the present interest in using viruses as expression vectors for vaccines. It has been suggested that adenovirus be used as such an expression vector by inserting the gene encoding the desired immunizing antigen into the genome of an adenovirus from which the E3 region had been largely deleted (26, 35). The vaccine strains of Ad4 and Ad7 would be particularly valuable for this purpose since these strains have been shown to be avirulent when given orally (36, 37). It is unclear, however, whether the deletion of the E3 region would convert the mutated, avirulent virus to a virulent strain.

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