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The effect of latent adenovirus 5 infection on cigarette smoke-induced lung inflammation

T.Z. Vitalis*, I. Kern**, A. Croome*, H. Behzad*, S. Hayashi*, J.C. Hogg*

The effect of latent adenovirus 5 infection on cigarette smoke-induced lung inflammation. T.Z. Vitalis, I. Kern, A. Croome, H. Behzad, S. Hayashi, J.C. Hogg. ©ERS Journals Ltd 1998.
ABSTRACT: The aim of this study was to test the hypothesis that latent adenovirus (Ad) 5 infection increases the lung inflammation that follows a single acute exposure to cigarette smoke. A recently developed model of latent adenoviral infection in guinea-pigs was used.

Twelve animals were infected with Ad5 (10^8 plaque-forming units) and 12 animals were sham-infected. Thirty five days later six Ad5-infected and six sham-infected animals were exposed to the smoke from five cigarettes. The remaining animals were used as controls for both infection and smoking. As markers of inflammation, the volume fraction of macrophages, T-lymphocytes, neutrophils and eosinophils were measured by quantitative histology.

We found that latent Ad5-infection alone, doubled the number of macrophages in the lung parenchyma and that smoking alone, doubled the volume fraction of neutrophils in the airway wall and the volume fraction of macrophages in the lung parenchyma. Neither viral infection nor smoking, alone, had an effect on T-lymphocytes or eosinophils. However, the combination of viral infection and smoking doubled the T-lymphocyte helper cells and quadrupled the volume fraction of macrophages in the lung parenchyma.

We conclude that in guinea-pigs, latent adenovirus 5 infection increases the inflammation that follows a single acute exposure to cigarette smoke, by increasing the volume fraction of macrophages and T-lymphocyte helper cells.
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*The University of British Columbia Pulmonary Research Laboratory, St. Paul's Hospital, Vancouver, British Columbia, Canada. **Institute for Respiratory Diseases, Golnik, Slovenia.

Correspondence: T.Z. Vitalis
The University of British Columbia
Pulmonary Research Laboratory
St. Paul's Hospital
1081 Burrard St.
Vancouver
British Columbia V6Z 1Y6
Canada
Fax: 1 604 6315351

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Cigarette smoking causes airways inflammation in every-one [1] but only produces airways narrowing and ob-struction in 15–20% of heavy smokers [2]. This suggests that smoking must act in combination with other risk factors such as viral infection to produce airways obstruction [3]. Several studies from our laboratory have suggested a possible link between cigarette smoke-induced airway in-flammation and latent adenovirus (Ad) infection [4, 5]. We have shown that lung tissue samples taken from smokers with airways obstruction had three times more Ad E1A deoxyribonucleic acid (DNA) than those without obstruction [4] and that the E1A gene was expressed in epithelial cells in the airways and lung parenchyma [5].

The expression of E1A proteins in latent Ad infections is interesting as these viral proteins have the ability to bind to transcription factors [6] and consequently have the potential to alter the expression of host genes capable of driving the inflammatory process. For example, monocytic (THP-1) and T-lymphocytic (Jurkat) cell lines transfected with plasmids containing the 13 Svedberg (S) E1A coding and regulatory regions produce two and 21 times more tumour necrosing factor (TNF), respectively, when stimulated, compared to cells transfected with control plasmids [7]. In addition, pulmonary epithelial cells, permanently transfected with a plasmid containing the Ad5 E1A gene, show increased intercellular adhesion molecule (ICAM-1) [8] and interleukin (IL)-8 [9] expression when stimulated with endotoxin. These results suggest the possibility that cells expressing the E1A proteins produce increased amounts of pro-inflammatory molecules such as TNF, IL-8, and ICAM-1 when exposed to inflammatory stimuli and that this excess of cytokine production would increase the normal inflammatory reaction to such stimuli. On the basis of these results we hypothesise that the E1A protein can increase the inflammatory reaction *in vivo* in response to stimuli such as cigarette smoke.

To test the hypothesis that latent Ad infection increases the inflammatory reaction caused by cigarette smoke, a model of latent Ad5 infection which has been produced in guinea-pigs has been used [10]. The important features of the guinea-pig model of latent Ad5 infection are that: 1) the virus replicates and produces a pathology similar to that seen in human adenovirus infections (the acute phase); 2) Ad5 E1A DNA and protein can be localized to the lung at least 7 weeks after the cessation of viral replication (latent phase); and 3) that a chronic low grade bronchiolitis persists during this latent phase. In this study we will measure the inflammation caused by a single acute exposure to cigarette smoke in guinea-pigs with a latent Ad5 infection.

Materials and methods

Animals, viruses and cell culture

Female guinea-pigs (*Cavia porcellus*, 250–300 g) were purchased from Charles River Canada (St Constant, Q, Canada) and housed in polycarbonate rat cages fitted with high-efficiency particulate air (HEPA) filter covers. The animals were provided with food and water *ad libitum*. Wild-type Ad5 was obtained from the American Type Culture Collection (Rockville, Md, USA) and propagated in monolayer cultures of A549 cells grown in minimal essential medium (MEM) (Gibco BRL Life Technologies Inc., Gaithersburg, Md, USA) supplemented with 10% foetal bo-vine serum (FBS). Two to five days after inoculation with Ad5, cells were freeze/thawed twice, sonicated for 30 s three times and spun at 500×g for 5 min. The supernatant was collected and its viral titre was determined by plaque assay on A549 monolayers grown on six-well plates. Titres ranged 10^8–10^9 plaque-forming units (pfu)·mL^{-1}. Sham in-oculum was prepared from uninfected A549 cells treated in an identical manner to the infected cells.

Cigarettes and smoking

Plain-ended cigarettes purchased through the Canada Tobacco Manufacturers' Council yielding 16 mg tar, 1.1 mg nicotine and 11 mg carbon monoxide, under a standard smoking regimen when smoked to a 23 mm butt length, were used in the study. Animals were immobilized in a stall constructed of poly methyl methacrylate with the snout protruding through a rubber dam into a chamber (400 cc) attached to a smoking machine as previously described [11, 12]. Smoke from five cigarettes was delivered (20 puffs·$cigarette^{-1}$; 20 mL·$puff^{-1}$) over a 40 min period.

Study design and experimental protocol

The experiments were designed to determine the effect of infection, smoking, and the interaction between the two treatments on the amount of inflammation in the lung. Twelve guinea-pigs were randomized and infected intranasally under Halothane® anaesthesia with Ad5 (10^8 pfu) in 300 µL of culture media. Another 12 guinea-pigs were sham-infected with sterile A549 culture media alone to control for the effect of latent infection. Thirty five days later six infected and six sham-infected guinea-pigs were chosen randomly and exposed to cigarette smoke as described above. To control for the effect of smoking, the remaining six infected and six sham-infected guinea-pigs were placed in the smoking apparatus, but exposed to room air. After 6 h of smoke or room air exposure, animals were killed with an overdose of *i.p.* administered sodium pentobarbital. The lungs were removed and assessed for inflammation by quantitative histology.

Estimate of the volume of lung compartments and inflammatory cells by quantitative histology

After the animals were killed, the thoracic cavity was opened and the lungs inflated with a 1:1 mixture of optimal cutting temperature (OCT) compound (Tissue Tek; Miles Inc., Elkhart, IN, USA) and phosphate-buffered saline (PBS) (0.149 M NaCl, 0.012 M Na_2HPO_4, 0.004 M KH_2PO_4, pH 7.4). Next, the inflated lungs were removed and their volumes measured by a water displacement method to ±0.2 mL. Each lobe, except the right caudal lobe, was sectioned into six blocks in the transverse plane. Blocks were chosen in a systematic random fashion for freezing in liquid nitrogen, or for fixation in 10% buffered formalin. Tissue sections were coded in order to blind the observers to the experimental groups. The volumes of the lung compartments and of neutrophils, eosinophils, macrophages and T-lymphocyte subsets in airway wall and parenchyma lung tissue were estimated by point counting using a multi-level cascade design [13]. Total lung volume normalized to body mass was used as the reference volume from which the volume fractions of lung compartments and inflammatory cells were derived. A digital imaging system (Bioview, Infrascan, Richmond, BC, Canada) was used to overlay a point counting grid over the sections. The density of the point counting grid and the number of fields counted were adjusted to maintain the coefficient of error of the estimate of the volume ($CE_{est,V}$) below 0.10 [14]. The number of points falling on an object or region of interest within a field of view was counted and this number was divided by the total number of points in that field. The resulting fraction was multiplied by the lung volume to give a volume for that particular object or region of interest. The lung compartments of airspace, alveolar tissue, airway wall and blood vessels were determined at 40× magnification. The airway wall compartment included both the mucosa and the outer adventitia. In cartilaginous airways the cartilage volume was not included in the determination of the airway wall volume. Neutrophils and eosinophils were identified by their morphology on formalin fixed, paraffin embedded tissue sectioned (4 µm) onto glass slides and stained with haematoxylin and eosin, at 1,000× magnification.

Immunocytochemistry

T-lymphocyte subsets and macrophages were determined by immunocytochemistry staining. Frozen sections, 6 µm thick, were cut onto silanized slides, air dried and fixed in 4% paraformaldehyde in PBS for 10 min. Monoclonal mouse immunoglobulin (Ig)G antibodies specific for guinea-pig T-lymphocyte helper/inducer subset (clone CT7) analogous to human CD4 T-lymphocyte subset, T-lymphocyte cytotoxic/suppresser subset (clone CT6) analogous to human CD8+ subset and a cytoplasmic component of all guinea-pig tissue macrophages and monocytes (clone MR-1) were purchased from a commercial source (Serotech Ltd., Kidlington, Oxford, UK). Antibody binding was detected with the alkaline phosphatase anti-alkaline phosphatase (APAAP) method (Dakopatts, Glostrup, Denmark) and positively stained cells were identified at 200× magnification. For each section from a block stained and antibody used, nonspecific mouse IgG was used as a negative control.

Detection of the Ad5 E1A gene by polymerase chain reaction (PCR).

PCR was used to detect the E1A gene of Ad5 in DNA extracted from the lungs of guinea-pigs. The previously

frozen right caudal lobe was divided into half. From each half, frozen tissue was shaved and minced to a fine powder with a new, cold razor blade. Separate DNA extractions were carried out on each half. In order to remove the OCT the minced tissue was rinsed in PBS and centrifuged. The pellet was resuspended in digestion buffer (0.1 M NaCl, 0.01 M TrisHCl (pH 8.0), 0.025 M Na_2 ethyl-enediamine tetra-acetic acid (EDTA), 0.5% sodium dode-cylsulphate (SDS)), containing 0.1 mg·mL^{-1} proteinase K (Gibco BRL) and allowed to digest overnight at 50°C. The digested tissue was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) buffered with TrisHCl (pH 8.0) and the resulting aqueous phase was extracted with chloro-form. After ethanol precipitation, the DNA pellet was dis-solved in 200 µL TE (0.010 M TrisHCl, 0.001 M Na_2EDTA, pH 8.0). The DNA solution was then analysed by PCR for the E1A region of adenovirus. The PCR for Ad5 E1A was done on 10 µL of the extracted DNA. The oligonucleotide primers for the E1A PCR were nucleotides 768–790 (5'TAATGTTGGCGGTGCAGGAAGGG3') and nucleotides 1232–1253 (5'TCAGGCTCAGGTTCAG-ACACAG3') in the Ad5 genome. The size of the expected PCR product was 486 base pairs (bp). Amplification was performed in 100 µL reaction volumes containing 10 mM TrisHCl pH 8.3, 50 mM KCI, 200 µM each deoxyribonucleoside triphosphate (dNTP), 2.5 mM $MgCl_2$, 0.5 µM primers, 10 µL of the extracted DNA, and 2.5 units of Taq polymerase (Gibco BRL). The reaction mixture (minus the Taq polymerase) was overlaid with 80 µL of mineral oil and was subjected to heat at 80°C in a RoboCycler (Stratagene, La Jolla, CA, USA) and after 1 min 2.5 units of Taq polymerase were added. The following cycling parameters were applied in: 40 cycles of: 93°C for 1 min; 63°C for 1 min; 72°C for 2 min; and with an added 7 min at 72°C to the last cycle to ensure complete extension. All PCR reactions were accompanied by appropriate positive and negative controls. One hundred copies of Ad2 DNA (Bethesda Re-search Laboratories, Gaitersburg, Md, USA) were used as a positive control. Ad2 and Ad5 have homologous DNA sequences for the primers selected for E1A PCR. TE without template DNA and DNA extracted from sham-infected guinea-pigs were used as negative controls.

Analysis of the PCR products

One third of the PCR product was subjected to electrophoresis on 0.8% agarose gels and transferred to Hybond-N filters (Amersham, Arlington Heights, IL, USA). Southern hybridization using an E1A probe was performed as previously described [4].

Statistical analysis

Changes in volume fraction of lung compartments and inflammatory cells were analysed using two-way analysis of variance (ANOVA). The level of significance for the independent main effects of infection and smoking was $p<0.05$. Reported p-values for each cell type were corrected for five cell types in the airway wall and three cell types in the parenchyma for a total of eight analyses by the ranked Bonferroni method [15]. When interactions were suspected between infection and smoking then multiple comparisons between the means of the experimental groups were analysed using t-tests to determine if the interaction was one of interference or synergy [15]. The multiple comparisons made between experimental groups were as follows: infected/smoking *versus* uninfected/smoking; infected/nonsmoking *versus* uninfected/nonsmoking; infected/smoking *versus* infected/nonsmoking; and uninfected/smoking *versus* uninfected/nonsmoking. The level of significance was maintained at $p<0.05$ for the multiple comparisons by correcting for 12 analyses (eight analyses for the various cell types in the two lung compartments plus the four analyses for the multiple comparisons) by the Bonferroni method [15].

Results

One animal in the infected/smoking group died of undetermined causes after exposure to cigarette smoke.

PCR for the Ad5 E1A gene

A representative autoradiograph of a Southern blot hybridization for the E1A PCR product is shown in figure 1. Four of five infected/smoking animals and five of six infected/nonsmoking animals were positive for the E1A gene in at least one of the two DNA extractions from the right caudal lobe of each animal. None of the sham-infected animals were positive for Ad5 E1A DNA by PCR in either of the two extracted DNAs.

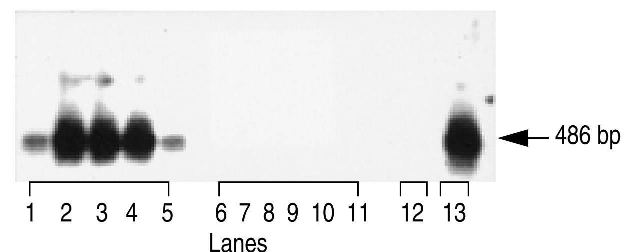

Fig. 1. – Representative autoradiograph of Southern blot hybridization of E1A gene polymerase chain reaction products from infected and sham-infected guinea-pigs probed with adenovirus (Ad) E1A deoxyribonucleic acid (DNA). Lanes 1–5: infected; Lanes 6–11: uninfected; Lane 12: no template; DNA control; Lane 13: 100 copies Ad2 DNA.

Table 1. – The volume of lung compartments of guinea-pigs in the experimental groups

	Uninfected nonsmoking (n=6)	Infected nonsmoking (n=6)	Uninfected smoking (n=6)	Infected smoking (n=5)
Body mass	0.566	0.543	0.529	0.456
kg	(0.010)	(0.012)	(0.007)	(0.014)
Lung volume	46.60	44.67	42.98	50.47
mL·kg BM^{-1}	(2.24)	(1.98)	(1.28)	(2.43)
Air space	25.27	24.63	24.74	29.83
mL·kg BM^{-1}	(1.54)	(1.82)	(0.87)	(1.24)
Alveolar tissue	16.79	16.31	13.18	15.68
mL·kg BM^{-1}	(0.54)	(1.13)	(0.81)	(1.57)
TAW	1.18	1.17	1.21	1.27
mL·kg BM^{-1}	(0.09)	(0.10)	(0.06)	(0.07)
Blood vessel	3.34	2.76	3.72	3.73
mL·kg BM^{-1}	(0.37)	(0.22)	(0.33)	(0.66)

Values are presented as mean with SEM in parenthesis. BM: body mass. TAW: total airway wall.

Lung and airway compartment volumes

The air, alveolar tissue, blood vessels, and airway wall represented 57, 33, 7 and 3% of the total lung volume including the airspace, respectively (table 1). The volumes of these lung compartments were not significantly different between the four experimental groups.

The volume of inflammatory cells in the lung parenchyma (table 2)

The lung parenchyma included the air space and its surrounding tissue and blood vessels but excluded the airway wall. Polymorphonuclear cells were not determined in the parenchyma compartment because of the difficulty in using morphological criteria to identify these cells in interstitial tissue.

Immunocytochemistry, however, provided easy identification of macrophages and the T-lymphocyte subsets (table 2). Viral infection alone caused a doubling of macrophage volume (p=0.01). Smoking alone also caused a two-fold increase in macrophage volume (p=0.02). The effects of these two treatments were independent of each other. As a result of the two treatments together, infected animals exposed to smoke had a four times greater volume of macrophages in the lung parenchyma than uninfected animals not exposed to smoke (fig. 2a). For CD4+ T-lymphocytes in the parenchyma there was an interaction between the infection and smoking. Multiple comparisons

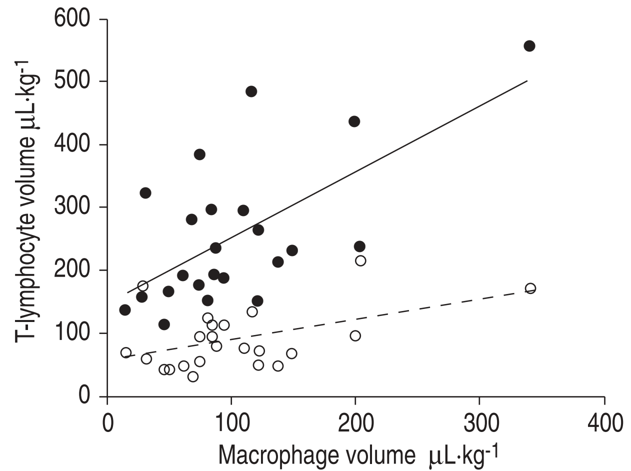

Fig. 3. – Linear regression analysis of CD4+ volume as a function of macrophage (Mac) volume in the lung parenchyma. (CD4+ (µL·kg-1)= 0.98Mac (µL·kg-1) + 154 (µL·kg-1); p=0.001; r^2=0.42; (—●—) and CD8+ volume (CD8+ (µL·kg-1)=0.30Mac (µL·kg-1)+61 (µL·kg-1); p=0.019; r^2= 0.237; - -○- -).

Table 2. – The volume of inflammatory cells in the lung parenchyma. Volume of inflammatory cells analysed with a two way analysis of variance (ANOVA)

	Uninfected nonsmoking (n=6)	Infected nonsmoking (n=6)	Uninfected smoking (n=6)	Infected smoking (n=5)	ANOVA Infection p-value	ANOVA Smoking p-value
Mac µL·kg-1	44.7 (10.4)	105.5 (22.5)	90.5 (10.1)	191.3 (47.2)	0.01*	0.02*
CD4+ µL·kg-1	196.8 (27.0)	190.0 (27.0)	267.2 (28.6)	393.4 (62.7)	0.72	0.01**
CD8+ µL·kg-1	83.3 (19.7)	96.0 (26.8)	82.4 (13.6)	109.3 (19.5)	0.97	0.75

Values are presented as mean with SEM in parenthesis. The volume of inflammatory cells was analysed using a two-way analysis of variance (ANOVA). Mac: macrophage. Bonferroni correction was applied to the p-values. *: significant main effect; **: significant interaction. For further explanation refer to figure 2.

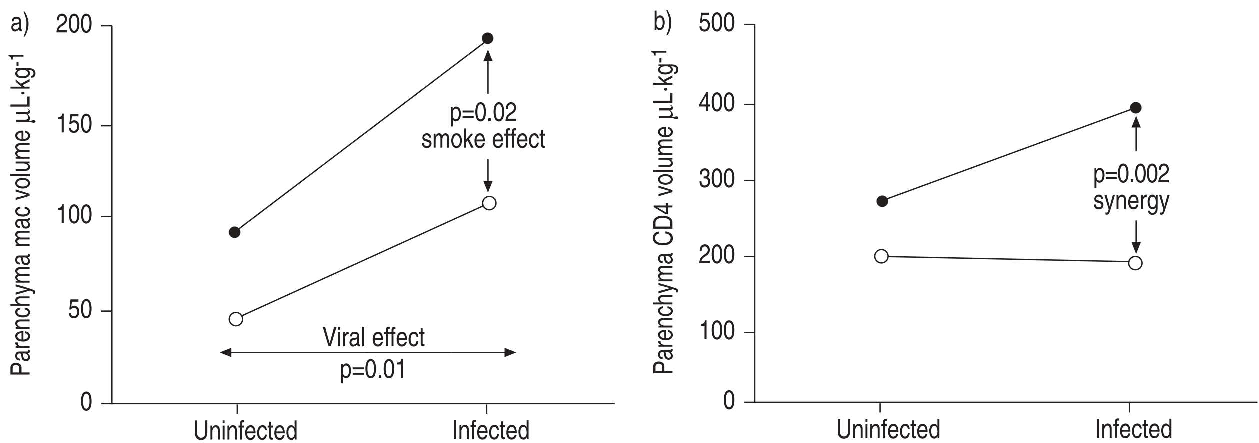

Fig. 2. – Profile plot of experimental group means for: a) macrophages (mac); and b) CD4+ cells in the lung parenchyma from two-way analysis of variance. The effects of infection (p=0.01) and smoking (p=0.02) were independent and additive for macrophage volume in the lung parenchyma. The combination of viral infection and smoke was synergistic (p= 0.002) for CD4+ cells in the lung parenchyma. All reported p-values are corrected for multiple comparisons by the Bonferroni method. —○—: no smoke; —●—: smoke.

Table 3. – The volume of inflammatory cells in the airway wall

	Uninfected nonsmoking (n=6)	Infected nonsmoking (n=6)	Uninfected smoking (n=6)	Infected smoking (n=5)	ANOVA	
					Infection p-value	Smoking p-value
Eosin µL·kg-1	50.2 (4.4)	72.4 (6.9)	59.0 (4.9)	50.4 (7.5)	0.92	1.00
Neut. µL·kg-1	0.7 (0.1)	1.3 (0.5)	2.1 (0.3)	2.5 (0.3)	0.65	0.01*
Mac µL·kg-1	10.2 (2.0)	12.2 (2.1)	9.0 (1.6)	15.6 (1.4)	1.00	0.15
CD4+ µL·kg-1	4.2 (1.2)	1.7 (0.5)	4.0 (0.4)	5.2 (1.2)	0.45	0.49
CD8+ µL·kg-1	4.4 (0.8)	4.4 (0.8)	2.8 (0.7)	4.2 (0.8)	1.00	0.68

Values are presented as mean with SEM in parenthesis. The volume of inflammatory cells was analysed using a two-way analysis of variance (ANOVA). Eosin: eosinophil; Neut.: neutrophil; Mac: macrophage. Bonferroni correction was applied to the p-values. *: significant main effect.

showed that CD4+ T-lymphocytes were increased significantly only in infected/smoking animals when compared with infected/nonsmoking animals (p=0.002) indicating that the interaction between viral infection and smoking was synergistic (fig. 2b). There was no significant change in CD8+ volumes between the experimental groups.

Linear regression analysis of the combined data for all experimental groups showed a significant relationship between CD4+ T-lymphocytes and macrophages indicating that 42% of the variation seen in CD4+ volume could be predicted by the variation in macrophage volume (fig. 3). A significant but weaker relationship between CD8+ T-lymphocytes and macrophages was detected where 24% of the variation in CD8+ volume could be accounted for by the variation in macrophage volume (fig. 3). There was no significant correlation between CD4+ and CD8+ volumes.

The volume of inflammatory cells in the airway wall (table 3)

Eosinophils were abundant in the airways but their numbers were not significantly affected by smoking or viral infection. On the other hand, smoking significantly increased the volume of neutrophils in the walls of airways of both the infected and uninfected groups (p=0.01) but viral infection had no effect. When cyrosections were analysed for macrophages and T-lymphocyte subsets no effect of smoking or viral infection was detected after the Bonferroni correction for either cell type (table 3). Linear regression analysis showed no significant relationship between macrophage volume and the volume of the T-lymphocyte subsets in the airway wall.

Discussion

Latent viral infection as a result of a single acute exposure to cigarette smoke had a significant impact on the inflammatory response seen in guinea-pigs, with increased numbers of macrophages and helper/inducer T-lymphocytes in their lung parenchyma. The effects of Ad5 infection and of smoking on the number of macrophages in the lung are independent of each other. Guinea-pigs receiving both treatments had a fourfold increase in the number of macrophages compared to control animals. The observed accumulation of macrophages in the infected/nonsmoking group may be due to the persistence of these cells after being recruited to the lung during the acute phase of the viral infection. Smoking further increased the number of macrophages possibly because macrophages present due to the acute phase of the infection responded to smoke by releasing chemokines that recruited monocytes from the peripheral blood [16–19].

The combination of smoking and latent viral infection had a synergistic effect on helper/inducer T-lymphocyte (CD4+) recruitment such that infected guinea-pigs exposed to cigarette smoke had twice the amount of CD4+ cells. This synergistic response may result from either a primary effect of the E1A gene on CD4+ cells, or a secondary effect of the acute phase of the viral infection on macrophage numbers, or a combination of the two. A possible example of a primary effect of the E1A gene on T-lymphocytes would be an alteration in cytokine expression. As T-lymphocytes transfected with Ad5 E1A have been shown to release more TNF upon stimulation *in vitro* [7] we speculate that similar responses to smoke exposure could occur *in vivo* and thus initiate pro-inflammatory, positive-feedback loops. The alternative is that the increase in CD4+ cells is secondary to and dependent on the number of pre-existing macrophages that have accumulated in the lung as a consequence of an acute viral infection. This possibility is supported by the significant dependence of CD4+ volume on macrophage volume, as shown in this study. Similar relationships between T-lymphocytes and macrophages have been shown in humans with emphysema [20] and in mice infected with *Cryptococcus neoformans* [21]. Finally, a combination of primary and secondary effects is implicated, as only 42% of the variation in CD4+ cells could account for the variation in macrophages.

Neutrophils and eosinophils were only measured in the airway wall tissue. Smoking caused a two- to threefold increase in neutrophils in the wall of the airways confirming the results of our previous studies [11]. The failure to demonstrate a difference between the infected and uninfected groups suggests that the E1A gene may not have persisted in the cells of the airway wall. In all four experimental groups, eosinophils were more prevalent than

neutrophils in the airway wall. This can be attributed to the high constitutive expression of eotaxin in guinea-pig lungs [22]. The number of eosinophils, however, was not affected significantly by the experimental treatments.

Studies in humans indicate that it is only the early genes of the adenovirus that persist whereas the late genes coding for viral structural proteins do not [4, 23]. It is not certain how long the early genes persist or how long their ability to increase lung inflammation lasts. In guinea-pigs, the E1A gene and resultant protein is present in the lungs for at least 7 weeks after resolution of the acute infection and is associated with low level chronic inflammation in the absence of viral replication [10]. It is not known if this condition lasts for the life-time of the guinea-pig or whether it slowly wanes as the lung tissue turns over with age.

In conclusion, these studies show that in guinea-pig lungs, latent adenovirus-5 infection increases the inflammatory response to an acute exposure to cigarette smoke by increasing the numbers of macrophages and helper T-lymphocytes. The exact nature of the interaction between latent infection and cigarette smoking in guinea-pigs remains to be determined. The interaction could involve primary effects of persistent adenovirus-5 E1A on the transcription of pro-inflammatory cytokines similar to those described in epithelial cells, lymphocytes and monocytes where transfection with E1A results in the upregulation of intercellular adhesion molecule-1 [8] and interleukin-8 [9], and tumour necrosing factor (TNF)-α [7], after challenge. Secondary effects residual to the acute viral infection could also play an important role in increasing the inflammation caused by cigarette smoke exposure. Further experiments are required to determine whether the virus-induced increase in the inflammation caused by cigarette smoke is prolonged by a chronic smoking regimen and whether this increase plays a role in the progression to obstructive lung disease.

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